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Equine Herpesvirus Type 9 in Giraffe with Encephalitis

To the Editor: Herpesviruses have been isolated from many mammals. Herpesvirus infection in natural hosts is often mild and is usually followed by a latent infection; however, cross-species herpesvirus infections cause severe and fatal diseases. Equine herpesvirus (EHV)–1 causes abortion, respiratory disease, and, occasionally, neurologic disorders in horses. EHV-1 infection is usually limited to equine species, although it has also been found in other species (*1*), in which it causes fatal encephalitis. Recent sequence analyses suggested that the equine herpesviruses isolated in the United States from onagers (*Equus hemionus*), Grevy's zebras (*E. grevyi*), and Thomson's gazelles (*Gazella thomsoni*) are a subtype or variant of EHV-1 (*2*). With respect to epizootiology, the nonequine animals affected by EHV-1 or EHV-1–related virus were kept in enclosures adjacent to those of zebra species (Grevy's or Burchell's).

Another EHV-related virus was isolated from 2 Thomson's gazelles that had encephalitis and were kept with zebras (*3*). The virus was later found to be a new type of EHV, EHV-9, although it was serologically cross-reactive with EHV-1 (*3*). Recently, neutralizing antibodies against EHV-9 were found among Burchell's zebras in the Serengeti ecosystem (*4*).

A herpesvirus was recently isolated from a reticulated giraffe (*Giraffa camelopardalis reticulate*) with neurologic symptoms; the giraffe was from a zoo in the United States (*5*). Nonsuppurative encephalitis was found by histopathologic examination of the giraffe brain. Several Burchell's zebras that were apparently healthy and later determined to be seropositive for EHV-1 were housed in the same pen as the giraffe. The isolated virus was identified by PCR and a monoclonal antibody assay as EHV-1 (*5*). In the present study, we analyzed 4 gene sequences of the giraffe herpesvirus to show its relatedness to EHV-1 and EHV-9.

We amplified portions of 4 genes from giraffe herpesvirus DNA by PCR. The DNA polymerase catalytic subunit (open reading frame [ORF] 30) gene was amplified by using herpesvirus universal primers (*6*). The genes for glycoprotein B (gB) (ORF33), glycoprotein 2 (gp2) (ORF71), and glycoprotein D (gD) (ORF72) were amplified by using primers specific for EHV-9. The ORF33 primers were gB-F (5′-GGCACAATAGTCCTAGCATG TCTGTTGCTG-3′) and gB-R (5′-AAATATCCTCAGGGCCGGAAC TGGAAAGTG-3′). The ORF71 primers were gp2-F (5′-CCCCGTTGATG AGTTTTGCGTAGAGGTCTA-3′) and gp2-R (5′-GCCACCACTGGTTG TAAAGGCCAAGAGATA-3′). The ORF72 primers were gD-F (5′-TTTACAACCACTGGTGGCGT GTGTGCAGAA-3′) and gD-R (5′-TATCTCCAAACCGCGAAGCTT-TAAGGCCGT-3′). The amplified products were used as templates for direct sequencing (Dragon Genomics, Mie, Japan). The sequences were edited with Phred, Phrap, and Consed (www.phrap.org/phredphrapconsed.html), and the phylogenic trees were constructed with PHYLIP (*2,7*). Accession numbers of the sequences (submitted to the DNA Data Bank of Japan) are given in the Figure.

We used PCR to amplify a part of the gB gene of the giraffe herpesvirus, and we used EHV-1 specific primers for sequencing. However, we could not obtain amplicons (data not shown). Therefore, the more conserved gene, ORF30, was sequenced. The sequence of the 1,066-bp segment of the giraffe herpesvirus ORF30 gene was 99.5% identical to EHV-9 and 94.6% identical to EHV-1, which indicates that the giraffe herpesvirus was most closely related to EHV-9. Therefore, EHV-9 ORF33–specific primers were used to amplify the corresponding region of the giraffe herpesvirus. The sequence of the giraffe herpesvirus ORF33 was 98.8% identical to EHV-9 and 95.9% identical to EHV-1. Also, the sequence of the other envelope glycoproteins (ORF71 and ORF72) of the giraffe herpesvirus were 99.8% and 99.6% identical to EHV-9 and 91.6% and 96.3% identical to EHV-1. A phylogenic tree of maximum likelihood showed that EHV-9 and the giraffe virus formed a genetic group that was apparently distinguished from other genetic groups of EHV (Figure).

Herpesviruses have caused clinical disease in zoo animals, including a case of EHV-9 infection in Thomson's gazelles (*3*) and a recently described endotheliotropic betaherpesvirus in-

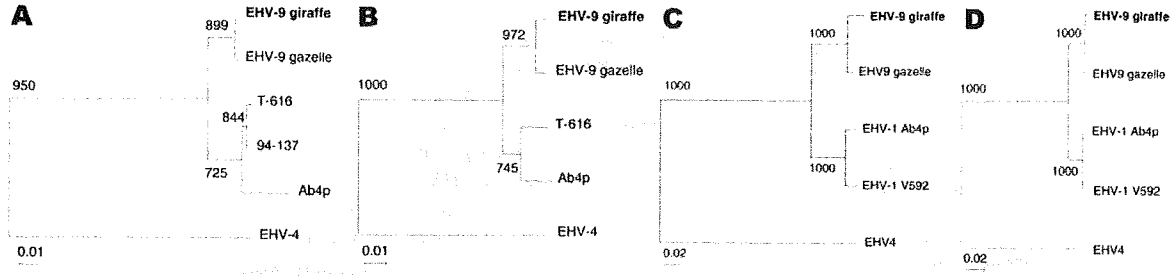

Figure. Phylogenic trees of giraffe herpesvirus and other related viruses. A) Open reading frame (ORF) 30, B) ORF33, C) ORF71, and D) ORF72. EHV-9 giraffe, equine herpesvirus (EHV) type 9 isolated from reticulated giraffe (*5*) (AB453826); EHV-9 gazelle, EHV-9 isolated from a Thomson's gazelle in Japan (*3*) (AP010838); T-616, EHV-1 isolated from a zebra fetus in the United States (EU087295); 94-137, EHV-1 isolated from a Thomson's gazelle in the United States (EU087297); Ab4p, EHV-1 isolated from horses (AY665713); EHV-4, EHV-4 isolated from horses (AF030027). Accession numbers of the sequences are AB439722 for ORF30, AB439723 for ORF33, AB453825 for ORF71, AB453826 for ORF72 of giraffe herpesvirus (DNA Data Bank of Japan, National Institute of Genetics, Japan), and AP010838 for EHV-9 genome sequence (H. Fukushi, unpub.data). Boldface indicates the sequence of EHV-9 derived from the giraffe. Scale bars indicate number of nucleotide substitutions per site.

fection in Asian and African elephants (*8*). The distribution and severity of herpesvirus encephalitis often differ between natural and accidental hosts in terms of enhanced neurovirulence. For example, herpes simplex virus causes a severe and fulminating encephalitis in rabbits but only herpetic stomatitis in humans; herpesvirus B infection is fatal to humans but not to other primates (*9*). These findings may explain why the giraffe had lesions while the zebras in the same enclosure did not.

Alphaherpesviruses can evade the immune system and become latent within lymphoid tissues, peripheral leukocytes, and trigeminal ganglia; they have the potential for reactivation and shedding after immune suppression or stress (*10*). Thus, the fact that the zebras were apparently healthy and seropositive for EHV-1 raises the possibility that the virus was reactivated and shed by one of the zebras, resulting in systemic infection and disease in the giraffe (*5*). This cross-species transmission of equine herpesviruses raises the possibility of latent infection and transmission of the disease from zebras to other animal species kept in zoos; the results could be devastating. Zebras might be EHV-9 carriers in zoos. Cross-species transmission must be considered in terms of screening susceptible animals for subclinical infection in terms of husbandry and housing issues for irreplaceable species.

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RESEARCH NOTES

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An Improved Method for Recovery of Muscle-Stage Larvae from Mice Infected with *Toxocara canis*

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ABSTRACT: We report a modified digestion method that improves the recovery of *Toxocara canis* larvae from skeletal muscle. Minced muscle tissue from infected mice was incubated in artificial gastric juice for 48 hr at 37 C, and ethanol was added for the second 24 hr. This procedure allowed the larvae to be identified and counted more quickly than with the standard digestion method. This method allows measurement of the total number of larvae present in muscle tissue following oral intubation of embryonated eggs, although it does not permit counting of live larvae.

?1

Following oral intubation of embryonated eggs, infectious-stage *Toxocara canis* larvae migrate into skeletal muscle tissue via systemic circulation. Muscle-stage larvae tend to increase in number after infection. Almost half of all recovered larvae enter skeletal muscles beyond the 10th day of infection (Oshima, 1961; Havasiova-Reiterova et al., 1995). These larvae are able to survive for long periods in muscle tissue. If an anthelmintic drug is effective against migrating larvae, the number of larvae appearing in skeletal muscle will be reduced. Therefore, for an anthelmintic trial, the number of muscle-stage larvae is a good indicator of efficacy (Fok and Kassai, 1998; Hrckova and Velebny, 2001; Horiuchi et al., 2005; Satou et al., 2005).

Both the Baermann technique and the digestion method using artificial gastric juice are used to detect larvae in skeletal muscle. The Baermann procedure, usually combined with a short-duration digestion method (less than 4 hr), permits the recovery of live larvae, but the extent of recovery is not satisfactory for estimating the total parasite burden. Additionally, since less than half of the skeletal muscle is usually employed for the digestion (Abdel-Hameed, 1984), the precise number of larvae recruited cannot be determined. In contrast, the digestion method alone permits a fairly good recovery, although a large amount of sediment remains after digestion, making the counting of larvae using stereoscopic microscopy quite time consuming. In the present report, we describe an improved method for recovering and counting larvae derived from skeletal muscle. The method is based on extended incubation in digestive fluid, followed by addition of alcohol.

Female BALB/c mice weighing 28–30 g were infected with 300 embryonated eggs of *T. canis* according to the method of Oshima (1961). Six mice were used for this experiment. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University. Three weeks later, skeletal muscle tissue from each mouse was minced with 150 ml of artificial gastric juice (0.5% of 1:10,000 pepsin and 0.7% hydrochloric acid, pH 1.5). After mixing well with a blender, the minced tissue was divided into 3 equal parts. The first portion was incubated in digestive fluid for 4 hr at 37 C with vigorous agitation. The mixture was then sieved with a wire mesh (mesh diameter: 1.0 mm), and the fluid was centrifuged at 320 *g* for 5 min. The total digestion time was 4 hr (method 1). Larval counting was performed on the resulting sediment using stereoscopic microscopy. Since undigested tissues remained on the mesh after sieving, these materials were re-incubated with digestive fluid for an additional 44 hr. They were vigorously agitated and prepared for counting in the same manner as before. The second portion of minced tissue was incubated in digestive fluid for 24 hr with vigorous agitation. The solution was centrifuged as before, and the sediment was re-incubated in 50 ml of fresh digestive fluid for an additional 24 hr. No filtration with wire mesh was performed. Thus, the total digestion time was 48 hr (method 2). Larval counts in the whole sediment were performed as before. The third portion was prepared in the same manner as the second portion, but 10 ml of 50% ethanol in distilled water was added to the sediment after the second 24 hr incubation step (method 3). The number of larvae in the sediment was then counted.

Table I shows the number of larvae recovered with each procedure. There was a significant difference in larval recovery between the 4-hr digestion group and the 48-hr digestion group ($p < 0.01$). Although ethanol treatment did not significantly affect recovery, we were able to find the larvae more easily in the ethanol-treated samples. The use of alcohol in the final step has the advantages that lipid droplets, which are insoluble in trypsin-based digestive fluid, are soluble in alcohol, and that alcohol acts as a surface-tension depressant that facilitates the identification of larvae. This is reflected in the time required to complete counting of a single sample: with ethanol treatment, counting took 16.7 ± 2.5 min (mean ± SD); without ethanol treatment, counting took 33.8 ± 7.5 min. For comparison, with the sample digested for 4 hr without ethanol, counting took 91.2 ± 14.1 min. From the undigested material, we were able to find larvae after additional incubation for 20 hr and 24 hr using freshly prepared digestive fluid, suggesting that a 4-hr incubation was insufficient for the digestion of skeletal muscle.

We further assessed whether this recovery technique can be carried out by an inexperienced person (T.N.). Six BALB/c female mice were orally administered albendazole (100 mg/kg/day) suspended in olive oil for 5 days, beginning 1 day before inoculation. Six control animals were given only olive oil. Three weeks after intubation, the mice were killed, and their skeletal muscle tissue was digested using method 3, under the guidance of an experienced researcher (Z.J.). Larvae migrating to the brain were counted by squash preparation (Abdel-Hameed, 1984). At the beginning of the experiment, it took almost 3 hr to complete the counting from just 1 skeletal muscle sample, but this soon fell to 30 min. The average recovery from skeletal muscle was 56.8 ± 4.8% in

TABLE I. Number of larvae recovered from skeletal muscle tissue of mice infected with 300 *T. canis* eggs.

Digestion period (hr)			
4		48	
		Ethanol treatment	
Sediment	Undigested material	No	Yes
9.5 ± 3.0	1 ± 0.9		
10.5 ± 3.7		23.2 ± 8.3*	26.3 ± 8.5*

Six mice were used for the experiment. Numbers are given as mean ± SD. Asterisk indicates a statistically significant increase in 48-hr incubation group versus 4-hr incubation group (Student's *t*-test, $P < 0.05$).

TABLE II. Number of larvae recovered from mice inoculated with 300 *T. canis* eggs.

Albendazole*		Control†	
Skeletal muscle	Brain	Skeletal muscle	Brain
50.7 ± 22.3	38.8 ± 12.9	104.5 ± 3.5	66 ± 11.8

Larval recovery from skeletal muscle was performed using method 3.
* Six BALB/c mice were treated with 100 mg/kg/day of albendazole suspended in olive oil for 5 consecutive days beginning 1 day before inoculation.
† Six control mice were given only olive oil.

the control group versus 29.8 ± 9.8% in the albendazole group. In skeletal muscle, 104.5 ± 3.5 larvae were found in the control group versus 50.7 ± 22.3 in the albendazole group, indicating that prophylactic treatment can reduce the larvae in skeletal muscle (Table II).

The improved method described here requires substantially less operator time (since it is more than 5-fold faster) to count larvae, and the recovery is 3-fold higher than that of our previously reported methods (Horiuchi et al., 2005; Satou et al., 2005). However, the larvae recovered are no longer alive, which is likely due to the much longer incubation time required. Therefore, while this method would be suitable for measuring the efficacy of treatments that act before larval migration, it would not allow measurement of the active larval tissue burden.

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INTERNAL MEDICINE

□ CASE REPORT □

Unusual Radiological Findings of *Fasciola Hepatica* Infection with Huge Cystic and Multilocular Lesions

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Abstract

This report describes a case of hepatic phase *Fasciola hepatica* infection presenting huge and multilocular lesions. The unique radiological findings mimicked hydatid diseases and also cystic liver neoplasm. Fascioliasis should be included in the differential diagnosis for cystic liver diseases.

Key words: *fasciola hepatica*, fascioliasis, eosinophilia, cystic, abscess, dot-ELISA

(Inter Med 47: 449-452, 2008)
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Introduction

Fascioliasis is a widespread infectious disease caused by trematode *Fasciola hepatica* (*F. hepatica*) infection (1). Although the radiological diagnosis of human fascioliasis has been improved, consideration of the possibility in the differential diagnosis is lacking in many developed countries. Typical computed tomography (CT) findings for hepatic phase of fascioliasis include small or sometimes clustered hypodense nodules and tortuous linear tracks, which are predominantly in subcapsular area (2, 3).

Here, we report a case with a unique hepatic phase fascioliasis. The patient was free from the symptoms, but presented uncommon radiological findings; a huge cystic lesion located in the middle of the liver together with peripheral multiloculated lesions.

Case Report

A 61-year-old Japanese man was referred to our hospital for the evaluation of migrating hepatic masses in November 2005. He had been involved in the construction of a power plant in Myanmar from January to November 2004. He had had a health checkup at a pre-consulted hospital in January 2005, and had undergone blood tests and abdominal ultrasonography (US) imaging. Although the clinical and laboratory findings were unremarkable except for peripheral blood eosinophilia (3,200/ml), the abdominal US imaging demonstrated multiple hypo-echoic lesions in right hepatic lobe. A contrast-enhanced CT scan showed multiple hypodense lesions in the right hepatic lobe. In the anterior segment of the right lobe, a huge and low attenuated mass measuring up to 57 mm with regular margins and some tiny hypodense lesions were detected (Fig. 1a, b). Thickening of the common bile duct or biliary dilatation did not exist. Although histological examination of the liver biopsy demonstrated the differentiatios from neoplastic lesions including intrahepatic cholangiocarcinoma or bile duct cystadenocarcinoma, the specimens were consistent with inflammation characterized by the presence of fibrotic changes and no sludge was drained. He was followed without any treatment and was referred to our hospital in November 2005.

On admission, physical examinations revealed only slight

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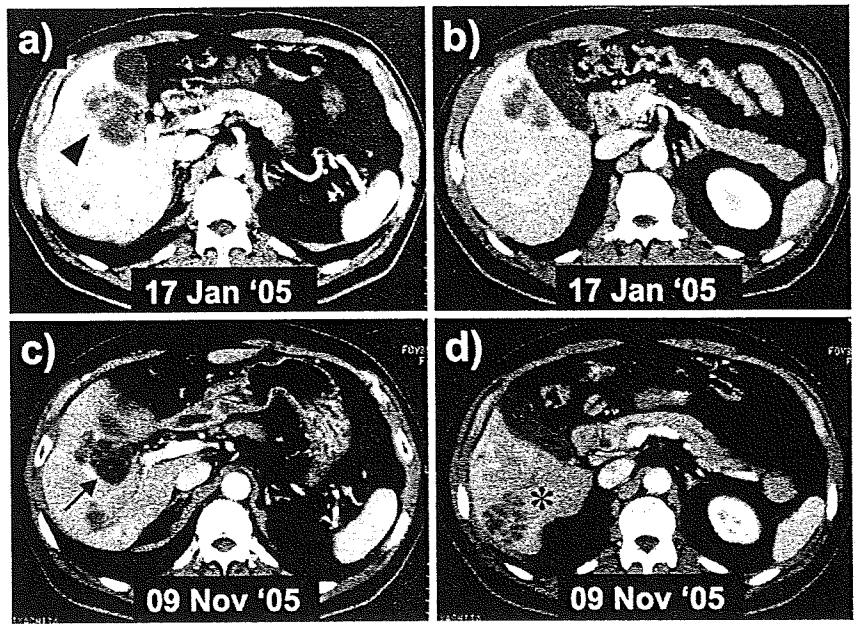

Figure 1. The contrast-enhanced CT image presented huge cystic and multilocular lesions. a, b) In the anterior segment of the right lobe, a huge and low-attenuated mass (arrowhead) and some tiny hypodense lesions were detected. c) The corresponding lesion in the anterior segment migrated into the center of the right lobe (arrow). d) A multilocular lesion (asterisk) was newly detected in the posterior segment of the right lobe.

hepatomegaly. Laboratory data showed the white blood cell count of 7,030/ml with a differential of 14.8% eosinophils. Serum IgE level was 438 U/ml (normal range; <250 U/ml). Neither ova nor larvae of any parasites were found in his stool. Contrast enhanced CT scans in November 2005, demonstrated mainly two types of masses in the right hepatic lobe. One of the masses, which had been detected in January but migrated during ten months, was located in the anterior segment and showed cyst-like hypodense lesion measuring up to 45 mm (Fig. 1c). The other mass, which could not be detected in January 2005, was located in the posterior segment and multiloculated (Fig. 1d). Because it was ineffective to distinguish between solid and cystic materials constructing these hypodense lesions with CT and ultrasonographic examinations, magnetic resonance imaging (MRI) was performed. The corresponding lesions proved to be hypointense on T1-weighted images (Fig. 2a), hyperintense on T2-weighted images (Fig. 2b, c), and extremely hypointensive foci on inverted diffusion-weighted images (Fig. 2d). These MR images suggested that these hepatic lesions consisted of necrotic or abscess-forming materials. MR cholangiopancreatography showed normal presentation.

The diagnosis was made by serologic tests. Because of the presence of eosinophilia and radiological changes of those lesions, we suspected that he suffered from some a type of parasitic infection. We conducted a screening test for parasitic antibodies in the patient's serum using a multiple dot enzyme-linked immunosorbent assay (dot-ELISA) (4). The antibody against *F. hepatica* was strongly positive by dot-ELISA. We also performed plate-ELISA and the ouchterlony double-diffusion test for confirmation. The ELISA titer for the antibody to *F. hepatica* was highly increased and the ouchterlony test showed a strong precipitin band against crude antigen of *F. hepatica* (Fig. 3). The antibody to *Echinococcus multiocularis* was negative in plate-ELISA. The patient was treated with triclabendazole (5). After 6 weeks, abdominal CT revealed a significant decrease in the size of the huge cystic lesion as well as the satellite lesions.

Discussion

F. hepatica is a trematode parasite that naturally infects cattle or sheep, and causes fascioliasis in almost every country around the world (1). Humans are an accidental reservoir host and could be infected by the ingestion of metacercaria-laden water plants. The infected young fluke, hatched from metacercaria, migrates in the peritoneal cavity and penetrates through the liver to the bile ducts causing acute hepatic phase of fascioliasis. In the later stage, the fluke matures and lodges in the bile duct resulting in chronic biliary disorder. In the acute hepatic phase, most patients note right upper quadrant pain, fever and malaise with eosinophilia, but a few cases remain asymptomatic like the case presented here (6). Although the diagnosis of fascioliasis is fundamentally made by the detection of the ova or fluke in the bile duct or stool, it is difficult to obtain such evidence until the patient advances to the chronic biliary phase.

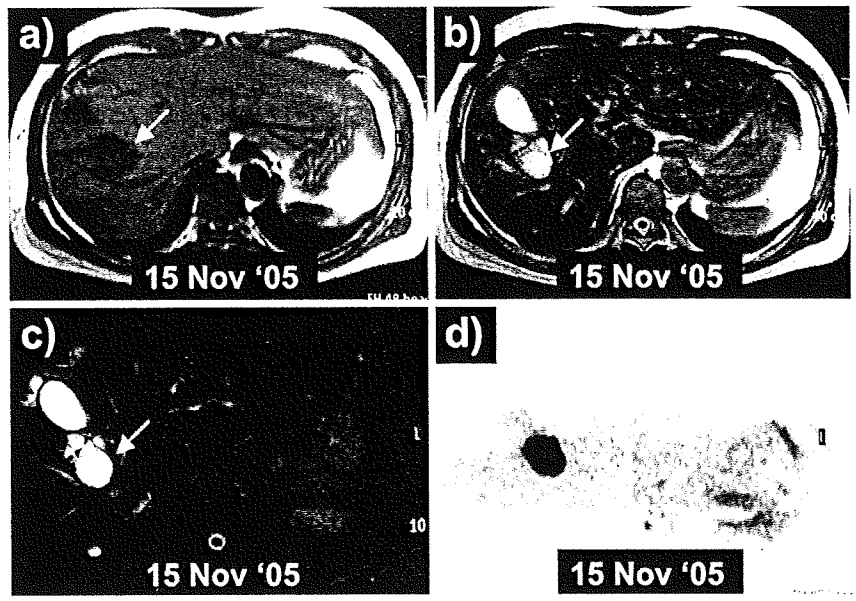

Figure 2. The corresponding MR image showed necrotic or abscess-forming lesions (arrows); a) T1-weighted images, b) T2-weighted images, c) fat-suppressed T2-weighted image, d) inverted diffusion-weighted images.

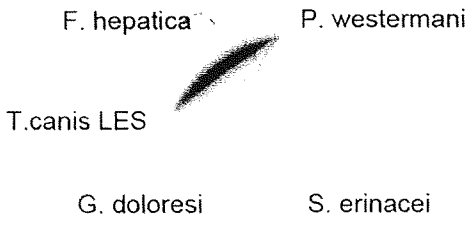

Figure 3. The ouchterlony double-diffusion test showed a strong precipitin band against crude antigen on Fasciola hepatica. The positions of antigens; F. hepatica, Fasciola hepatica; P. westermani, Paragonimus westermani; T. canis LES, Larval excretory and secretory antigen of Toxocara canis; G. doloresi, Gnathostoma doloresi; S. erinacei, Spirometra erinacei.

Typical CT findings for hepatic fascioliasis are nodular or tubular hypodense lesions up to 20-30 mm in diameter particularly in the subcapsular area (2, 7) because the infected form of metacercariae penetrates through the liver capsule and could cause subcapsular hemorrhage and frank hepatic necrosis before the biliary stage (8). However, some atypical radiographic findings have also been observed during acute or chronic fascioliasis (9, 10). In the present case, a huge abscess-forming lesion and asymptomatic physical presentations with eosinophilia mimicked hepatic unilocular hydatid disease (11) but that etiology is unknown. Usually, unilocular hydatid disease is caused by *Echinococcus granulosus* infection that produces unilocular and huge cystic lesions without any obvious symptoms. Although *Fasciola* and *Echinococcus* are quite different parasites, these parasitic diseases may present similar radiological appearances. Kim and colleagues reported confusing radiological findings of fascioliasis exhibiting huge abscess lesions without eosinophilia (9). That lesion was considered as an abscess-forming lesion with distinct thick wall and therefore as chronic biliary phase fascioliasis. These radiological and laboratory findings were not exhibited in the present case of hepatic phase fascioliasis; therefore our case was different from those in previous reports.

In the past decade, substantial progress in the radiological diagnosis of human fascioliasis has been achieved and some reports on the MR imaging have been well documented (12, 13). Cevikol and colleagues (12) reviewed the MR observations of hepatic fascioliasis and classified them into five types. In their article, hypointense lesions on T1-weighted images and brightly hyperintense lesions on T2-weighted images could be classified as one of the type of lesion. However, the appearance of the lesions in our case, i.e., huge and multiloculated masses, was not referred to as a usual pattern of hepatic fascioliasis. Intrahepatic cholangiocarcinoma or biliary cystadenocarcinoma could demonstrate the same signal patterns on T1-weighted and T2-weighted images, but the diffusion-weighted image is quite useful to distinguish solid neoplasms and necrotic cysts caused by fascioliasis as in the present case (14). Bacterial abscess also shows similar MR images, therefore, it is not possible to confirm the diagnosis based on MR images and thus examinations of other laboratory findings, serology and aspiration

specimens are necessary.

In conclusion, we emphasize here that hepatic fascioliasis can present a variety of lesions in the liver and huge cystic liver masses can also be produced. It is important to keep these findings in mind.

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INTERNAL MEDICINE

□ PICTURES IN CLINICAL MEDICINE □

Visceral Toxocariasis from Regular Consumption of Raw Cow Liver

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Key words: toxocariasis, visceral larva migrans (VLM), albendazole, mode of transmission, raw liver

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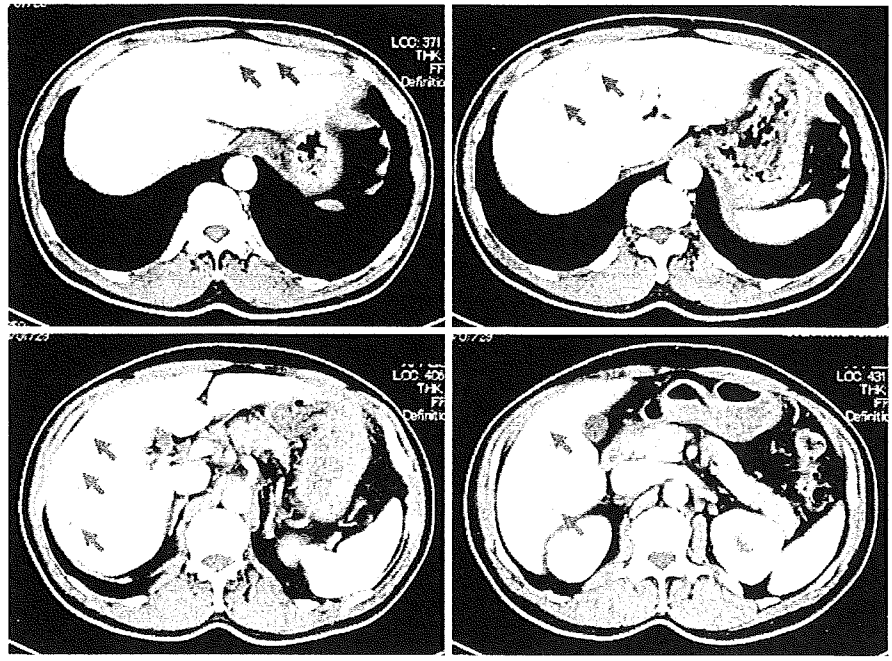

Contrast-enhanced CT scan image obtained at portal venous phase showing multiple small, ill-defined, and low-attenuation lesions in the liver of the patient (arrows).

A 58-year-old man had leukocytosis (leukocytes 11,800/μL), with marked eosinophilia (36%) and an increased total IgE at 2,345 U/mL (normal <100). There were no abnormal results in his annual check-up examinations including blood parameters until the most recent examination, when he began to eat raw cow liver weekly. Abdominal computed tomography (CT) revealed multiple, ill-defined, low-attenuated lesions in the patient's liver (Picture 1). Chest X-ray images did not reveal apparent abnormalities, whereas chest CT demonstrated a nodule with a halo and ill-defined margin, and ground-glass opacity (Picture 2). Gel diffusion test using the patient's serum revealed strong precipitin bands

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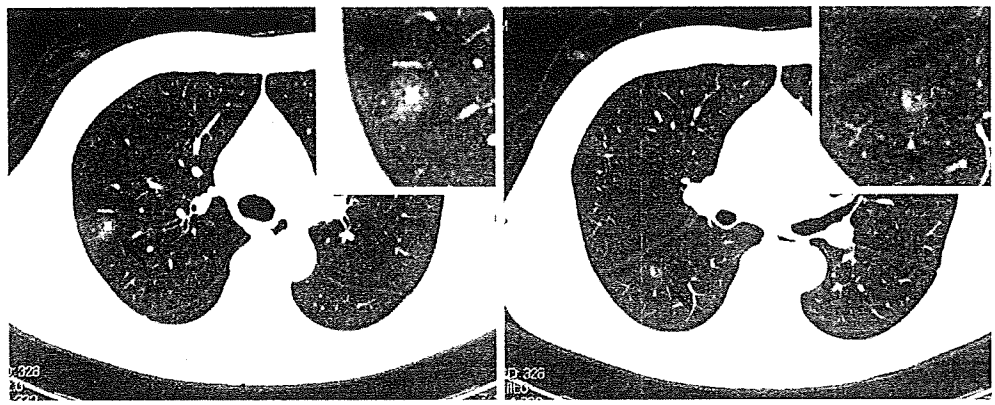

Picture 2. Chest CT scan image showing nodule with halo and ill-defined margin (left), and groundglass opacity with an ill-defined margin (right). The lesion is shown magnified in the inset.

against larval excretory-secretory (LES) products of both *Toxocara* (*T.*) *canis* and *T. cati*, thus visceral larva migrans (VLM) was highly suspected. Ttreatment with albendazole was performed. Consumption of paratenic meat, especially raw liver, was the suspected source of infestation.

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Prolactin evokes lactational transmission of larvae in mice infected with *Toxocara canis*

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ABSTRACT

We investigated the trans-lactational maternal–neonatal transmission of *Toxocara canis* larvae in mice, with particular interest in the role of prolactin in their migration to the mammary gland. Two female mice were infected with 300 *T. canis* eggs soon after delivery of 27 offspring. After 1 week of breast-feeding, seven larvae were recovered from 4 of 13 offspring. After 2 weeks of lactation, 101 larvae were recovered from all the remaining offspring. Daily prolactin administration (5 μg) was performed 2 weeks before *T. canis* infection and continued until 2 weeks after infection in six non-pregnant female mice, which resulted in larval accumulation in the mammary gland. Furthermore, prolactin administration in female mice that had been infected with *T. canis* 4 weeks prior to prolactin treatment induced migration of larvae into the mammary gland. These findings suggest that prolactin is a promoting factor contributing to lactational transmission of *T. canis* larvae in mice.

1. Introduction

Human larval toxocariasis is a serious public health problem in many countries [1]. Adult worms of *Toxocara canis* parasitize the intestines of domestic dogs and wild carnivores, and the larval stage of the parasite opportunistically invades undefinitive hosts including humans, resulting in human larval toxocariasis [2]. The migration behavior of the larvae in undefinitive hosts has been well documented [3–5]. In mice, *T. canis* larvae begin to accumulate in the liver 2 days post-infection, and they continue to migrate via systemic circulation. Beyond the 10th day of infection, most have settled in the brain and muscle tissue [6–8]. The larvae found in skeletal muscle are encapsulated in granulomatous inflammatory tissue and can survive for a long period [4,8]; those in the brain tissue elicit minimal inflammatory response [4].

Furthermore, it has been established that trans-placental transmission is the major route for *T. canis* larvae migration from infected female dogs to puppies [9–13]. In mice, it has also been regarded that *T. canis* larvae are transmissible via placenta [14–16], although no previous studies demonstrated larvae from offspring. Recently, Reiterova et al. [17] observed that *T. canis* larvae in offspring from infected mother mice were recovered at the beginning of the 5th day post-delivery. Thus, lactational transmission rather than trans-placental migration was certainly a possible route of maternal–neonatal infection with *T. canis*. After infection, migrating larvae settle in skeletal muscle tissue, in which they are then arrested in granulomatous inflammatory tissue. A re-emergence mechanism for these arrested larvae during pregnancy, however, has yet to be identified. In the present study, we demonstrate that *T. canis* larvae are able to transmit from mother to neonate via the mammary gland, and that prolactin evokes lactational transmission of the arrested larvae.

2. Materials and methods

2.1. Animals

Conventional ICR mice and an inbred strain of BALB/c mice were purchased from CLEA Japan Inc., Tokyo. All experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

2.2. Infections

T. canis eggs were obtained from the uteri of adult worms collected from naturally infected puppies after the administration of anthelmintics. Mature embryonated eggs were prepared following the method of Ohsima [5], and 300 eggs were inoculated into each mouse via a Teflon tube with a siliconized glass syringe [18].

2.3. Recovery of larvae

Each of the mammary glands and whole body of newborn mice were digested with artificial gastric juice (0.5% of 1:10,000 pepsin and 0.7% hydrochloric acid, pH 1.5) for 3 to 4 h with vigorous agitation. After centrifugation, the larvae in the sediment were counted using a stereoscopic microscopy on a microscope slide (7 × 14 cm). Examination

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Table 1
Numbers of larvae recovered from neonates

Mother mouse	7th day after birth				14th say after birth			
	Number of neonates examined	Number of neonates larvae recovered	Number of larvae/neonate	Total number of larvae recovered	Number of neonates examined	Number of neonates larvae recovered	Number of larvae/neonate[a]	Total number of larvae recovered
#1	5	4	1.4±0.5 (1–3)[a]	7	6	6	9.5±0.8 (8–13)[a]	57
#2	8	0	0	0	8	8	5.5±0.9 (2–10)[a]	44

Neonates were allowed to breast-feed from the mother mice, which were infected with 300 eggs of *T. canis* immediately after delivery.
[a] Mean±SD (range).

of the brain was performed according to the method of Cho et al. [18]. In this experiment, we attempted to recover the larvae from skeletal muscle tissue by using the digestion method described above. However, the results were inconsistent in the number of larvae recovered from adult mice, because a large amount of sediments remained after digestion, making the counting of larvae using stereoscopic microscopy difficult. Therefore, we omitted the data on the muscle-stage larvae of the adult mice in this experiment.

2.4. Pathology of the mammary gland

Mammary glands of female mice were removed and fixed in 10% neutral formalin solution. Serial sections were then prepared and stained with haematoxylin and eosin. The degree of eosinophil infiltration around the mammary gland was estimated by the number of cells per square millimeter. We randomly selected 10 fields with a microscope of 100-fold magnification. To confirm cell identification, we observed at high magnification and counted the number of eosinophils. A careful attention was paid not to shift the original position.

2.5. Experimental design for trans-mammary transmission of larvae

Two pairs of 8-week-old ICR mice were mated in separate cages until the female mice became pregnant. Within 12 h after delivery, each of two female mice was infected with 300 eggs of *T. canis*, and then allowed to breast-feed their offspring for 2 weeks. The offspring were divided into two groups: one was killed on day 7 after delivery, the other was killed on day 14 after delivery. The number of larvae in the offspring was counted using the digestion method described above.

2.6. Effect of prolactin treatment in non-pregnant, infected mice

To investigate the effect of prolactin on the stimulation of larval migration from skeletal muscle or brain tissue, eight BALB/c female mice, at 8 weeks of age, were intraperitoneally injected with 5 μg of prolactin (100 mg/mL, Sigma, St. Louis, USA) in physiological saline everyday for 14 days, and were then infected with 300 *T. canis* eggs orally. Prolactin treatment was then continued for another 14 days. After treatment, the mammary glands were removed and the larvae were recovered. Two mice were used for histological purposes. As a control, seven additional mice were administered 0.5 mL of saline instead of prolactin.

Table 2
Effect of prolactin treatment in non-pregnant infected mice

Treatment	Number of mice used	Number of mice larvae identified in mammary glands	Number of mice larvae identified in the brain	Number of larvae in mammary glands of identified mice	Number of larvae in the brain of identified mice
Prolactin	6	6	6	9.8±3.5[a]	36±16.3[a]
Saline	5	0	5	0	34.4±24.2[a]

[a] Mean±SD.

2.7. Effect of prolactin treatment in chronically infected mice

Six BALB/c female mice, at 4 weeks of age, were infected with 300 *T. canis* eggs. Four weeks later, 5 μg of prolactin was intraperitoneally administered everyday for 14 days. The mammary glands were then examined as described above. As a control, equal numbers of BALB/c mice were employed, and 0.5 mL of saline was injected into the peritoneal cavity everyday for 14 days.

2.8. Statistics

Statistical analysis was performed using Student's *t* test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Larval transmission to neonates via mammary gland after birth

Two mother mice delivered 11 and 16 offspring, respectively. The offspring from each infected mother mouse, which were infected with *T. canis* within 12 h after delivery, were randomly selected and sacrificed on day 7 or day 14 after delivery. Table 1 presents the number of offspring infected and the number of larvae recovered on each of these days. The rate of infection in the offspring and the average number of larvae recovered were higher in the group sacrificed on day 14 compared with that sacrificed on day 7. Additionally, the total number of larvae recovered was significantly higher in the day-14 group ($P<0.05$).

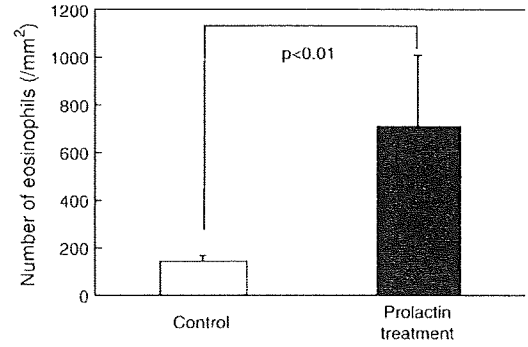

Fig. 1. Eosinophil counts around the capsules of mammary glands in mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 713.6±293.6 cells/mm² in the prolactin-treated group, and 144±21.3 cells/mm² in the saline-treated group. We randomly selected 10 fields with a microscope of 100-fold magnification. To confirm the cell identification, we observed at high magnification (×400) and counted the number of eosinophils. A careful attention was paid not to shift the original position.

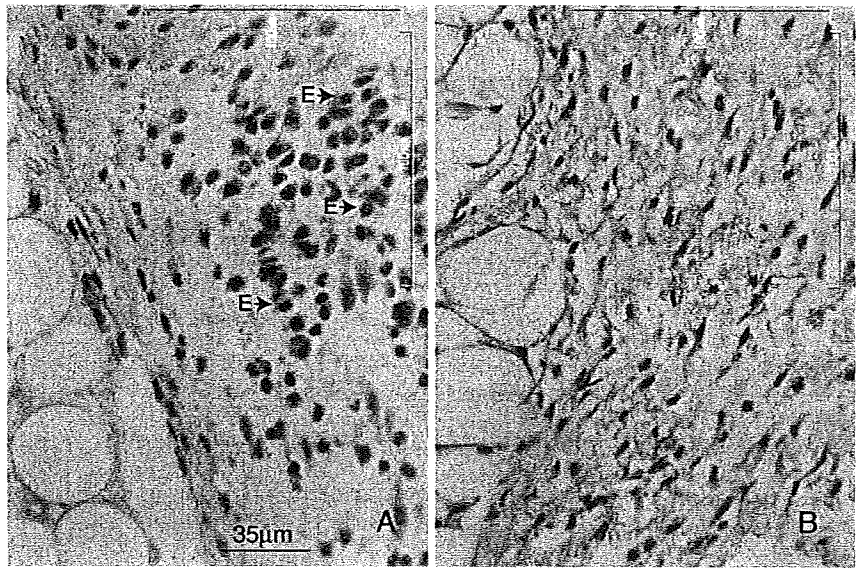

Fig. 2. Histopathological findings of mammary glands around the connective tissue in mice. Serial sections of mammary glands of female mice were stained with haematoxylin and eosin. Markedly higher eosinophilic (E) infiltrations around the connective tissue of the mammary gland were observed in the prolactin-treated mice (A) compared with the saline-treated mice (B).

3.2. Effect of prolactin on migration of larvae to the mammary gland

T. canis larvae were identified in the mammary glands of all infected mice, which were treated with 0.5 μg prolactin once a day intraperitoneally for 14 days before infection and 14 days after infection, although no larva was found in the control mice (Table 2). No significant difference in the number of larvae in the brain was observed between the prolactin-treated and saline-treated mice. These data suggest that prolactin might stimulate migration of larvae from skeletal muscle, the brain, or other organs to the mammary gland. Based on histological examination of 10 randomly selected fields, the eosinophil infiltrations around the capsule of the mammary gland were significantly increased in number in the prolactin-treated mice (713.6±293.6 6 cells/mm^2) compared with the saline-treated control mice (144±21.3 cells/mm^2, Figs. 1 and 2), suggesting that the inflammatory response against *T. canis* larvae was strong in the treated mice.

3.3. Effect of prolactin on chronically infected mice

Since administration of prolactin elicited a migration of larvae to the mammary gland, we next studied whether prolactin stimulates larval migration to the mammary glands from chronically infected mother mice in the absence of pregnancy. For this investigation, non-pregnant female mice, which had been infected with *T. canis* eggs 28 days previously, were administered prolactin for 14 days. Table 3 shows that larvae were recovered from the mammary glands in three of the four mice treated with prolactin, but no larva was found in the control mice. The number of eosinophils infiltrated in the mammary tissue was also significantly higher in the prolactin-treated group (Fig. 3).

In the prolactin-treated mice, glandular epithelial proliferation and dilatation of the ducts were observed, indicating a direct effect of prolactin against the mammary gland.

Table 3
Effect of prolactin treatment in chronically infected mice

Treatment	Number of mice used	Number of mice larvae identified in mammary glands	Number of mice larvae identified in the brain	Number of larvae in mammary glands of identified mice	Number of larvae in the brain of identified mice
Prolactin	4	3	4	3.8±1.9[a]	51.3±15.1[a]
Saline	4	0	4	0	49.8±5.7[a]

[a] Mean±SD.

4. Discussion

In this study, we demonstrate that *T. canis* larvae are able to migrate from the mother to neonates through suckling behavior, and that this migration can be induced by the administration of prolactin. While trans-placental migration of the larvae from female dogs to puppies has been established [9–13], few studies have investigated maternal–fetal transmission of the larvae in mice. Lee et al. [16] found that the larvae migrated in the uterus and placenta from the 9th day of pregnancy, and in the fetus from the 11th day of pregnancy when mother mice were infected during pregnancy. In addition, they

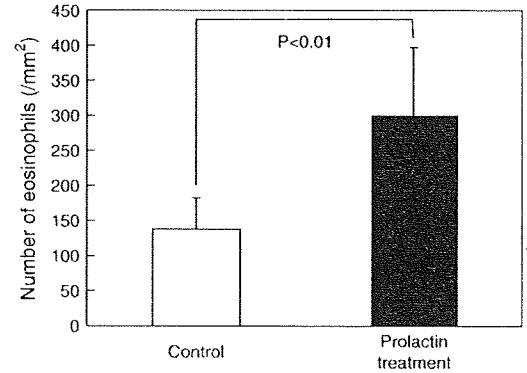

Fig. 3. Eosinophil counts around the capsules of mammary glands in chronically infected mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 300.8±95.6 cells/mm^2 in the prolactin-treated group, and 137.6±44.1 cells/mm^2 in the saline-treated group. Ten randomly selected fields at 100-fold magnification were observed via microscopy under a high magnification (400×).

identified larvae in the placenta and fetal blood vessels, histopathologically. They concluded that *T. canis* larvae were able to migrate through the placenta during pregnancy. However, because they did not examine the neonates after birth, they could not eliminate the possibility of trans-lactational transmission of the larvae from mother to neonates after delivery.

It is well documented that malaria infection induces placental injury, resulting in fetal loss in both humans and mice [19,20]. In murine toxocariasis, the litter sizes from infected mice are smaller than those from uninfected controls [21,22]. These data suggest that *T. canis* infection in mice can lead to mechanical injury of the placenta and a resultant decrease of litter size when the infection occurs during pregnancy.

Yet, in spite of these difficulties, newborns are still successfully delivered in most cases. In another previous study, larvae were found in offspring on day 5 after birth [15], suggesting suckling behavior might cause maternal–newborn transmission of *T. canis* larvae. In fact, our preliminary experiment revealed that larvae were first identified in offspring 11 days after birth (unpublished data). Thus, we hypothesized that larvae could migrate from mother to newborn mice through the mammary gland during suckling. The present findings support this hypothesis.

In general, *T. canis* larvae in mice settle in the brain and skeletal muscle after migration through the systemic circulation, and survive for a long period [4,8]. However, because we could not find any larvae in the mammary gland of non-pregnant infected mice, the larvae must be aroused by some sort of stimuli in order to migrate from those organs to the mammary gland. Prolactin, a lactogenic hormone, plays an essential role in the development of breast tissue. None of the non-pregnant mice not treated with prolactin showed the presence of larvae, in either the acute or chronic stage of infection, whereas prolactin-treated mice exhibited *T. canis* larvae infection in the mammary glands. One previous study discussed the relationship between *T. canis* infection and prolactin [23], reporting that the administration of prolactin led to a reduction in the number of larvae in infected mice. This may be related to the finding that prolactin acts as an immunomodulatory agent or proinflammatory cytokine in autoimmune diseases [24], and in several parasitic infections [25–28].

Eosinophil infiltration is a common feature in tissue-invading nematode infections, such as gnathostomiasis and trichinosis [29]. In toxocariasis, an eosinophilic granulomatous response is a typical pathological finding both in humans and in experimentally infected animals including mice [30,31]. Furthermore, eosinophil infiltration was demonstrated not only in the tissue adjacent to the larvae but also in that through which the larvae had passed [32]. These pathological changes are thought to be stimulated by the metabolic products from the larvae [29]. Therefore, we assumed that eosinophil infiltration around the capsule of the mammary gland in the prolactin-treated mice might be attributable to the migration of larvae into the mammary gland following stimulation of the tissue-arrested larvae.

The mechanism of this stimulation of tissue-arrested larvae during breast-feeding has yet to be elucidated. In hookworm infection, tissue-arrested larvae of *Ancylostoma caninum* were activated *in vitro* by TGF-β [33]. No such connection, however, has been demonstrated in *Toxocara* infection. The secretion of TGF-β is tightly regulated by the hormones estrogen and prolactin, and they are critical factors in the tissue-specific regulation of the local production of TGF-β in the mammary gland of the rat [34]. Therefore, we presumed that a similar cytokine reaction could be induced by prolactin, and may contribute to the reactivation of cryptic larvae in *Toxocara*-infected mice.

In the present study, we found clear evidence that prolactin is one of the factors in the lactational transmission of *T. canis* larvae from mother mice to offspring. Further investigation is needed to elucidate the precise mechanism of the stimulation of tissue-arrested larvae in mice.

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原　著

国産狂犬病ワクチンの皮内接種によるヒトへの狂犬病曝露前免疫の検討

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目　　的

2006年11月に国内で相次いで輸入狂犬病患者が発生した後[1,2)]，狂犬病ワクチンの需要が急増した．しかし，需要の増加が供給を上回っていたため，全国的に狂犬病ワクチンが品不足に陥った．このため，厚生労働省は緊急避難的に狂犬病曝露前免疫を制限する方針を示した．世界保健機関(WHO)は，狂犬病流行地において動物による咬傷を受けた場合，抗狂犬病免疫グロブリン(RIG)の投与と組織培養不活化ワクチン接種による曝露後発症予防を勧告している[3)]．ただし，曝露前免疫を受けていればRIGの投与が不要になる．RIGは世界的に不足しており，入手が容易でないため，曝露後免疫の効果を確実にする上で，狂犬病曝露前免疫を行っておくことが重要である．わが国における狂犬病ワクチンの生産量は少なく，急な増産ができないことを鑑みれば，今回のようなワクチン不足の事態に備えて，接種ワクチン量が少なくとも，高い効果を上げることができる接種法を検討しておくことが必要である．

狂犬病常在地であるタイでは，１人当たりの狂犬病ワクチン接種量を減量するために，皮内接種法(タイ赤十字方式)が広く用いられ，曝露後発症予防に効果を上げているばかりか[4)]，曝露前免疫にも採用され成果を上げている．国産の狂犬病ワクチンを用いての皮内接種法については，髙山らの皮内・皮下併用法以外に，その有効性や安全性がこれまで検討されておらず[5,6)]，皮内接種のみによる曝露前免疫は１例が報告されているにすぎない[7)]．

われわれは，国産ワクチンを用いた皮内接種法の可否を知るため，同意を得た健康成人に対して，国産狂犬病ワクチンを皮内接種して，狂犬病曝露前免疫の効果と安全性を調査した．

対象と方法

１．対　　象

本調査の目的，調査項目，接種ワクチンと予測される副反応について文書，および口頭で説明をして，同意が得られた医療・獣医療関係者17例を対象とした．

２．接種ワクチン

化学及血清療法研究所(化血研)製組織培養不活化狂犬病ワクチンのロットRB02およびRB03を用いた．狂犬病ワクチンは溶解液１mLで溶解した後，その0.1 mLずつを左右前腕に皮内注射した．

３．局所および全身反応

全例について，皮内接種15分後の接種局所における膨疹，発赤を視診で確認し，瘙痒感の有無を質問した．さらに，次回接種時および採血時に前回注射による局所の腫脹，発赤，疼痛，瘙痒感の自覚症状の有無について問診した．

４．抗体検査

狂犬病ワクチンを２～４週間隔で２回皮内接種し，２回目接種２～３週間後に採血して，血中抗狂犬病抗体価を測定した．血中抗狂犬病抗体価は，化血研臨床検査センターに依頼して，Platelia® rabies kit(BIO-RAD Laboratories)を用いてELISA法で測定した．

1）東京都立駒込病院感染症科　2）東京都立駒込病院小児科

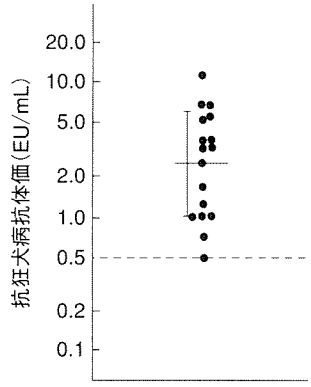

図1　被験者の抗狂犬病抗体価の分布

国産狂犬病ワクチン0.1mLを左右前腕に2～4週間隔で2回皮内接種した．2～3週間後の抗狂犬病抗体価(●)をELISA法で測定した．

横棒は幾何平均値，横棒を貫く縦棒は±SDを示す(n＝17)．

横点線はWHOの定める発症防御レベルを示す．

結　　果

1．対象者の年齢分布

対象者は男性11例，女性6例の合計17例であった．年齢分布は22歳から55歳まで，20歳代が13例，30歳代が2例，40歳代が1例，50歳代が1例であり，平均年齢は30.2±7.5歳であった．

2．血中抗狂犬病抗体価

ワクチンを2回皮内接種した2～3週間後には，17例全例で，抗体価が0.5 EU/mL以上に上昇していた．抗体価の範囲は0.5～11.5 EU/mL，幾何平均値は2.5 EU/mLであった(図1)．

3．接種後の局所反応および全身症状

ワクチン接種15分後，局所の発赤を呈した者は5例，腫脹を認めた者は9例，疼痛を認めた者は0例，瘙痒感を認めた者は2例であった．局所の発赤は，数日間残ったと報告した者があったが，発熱，頭痛，倦怠感などの全身症状を報告した例はなかった．

考　　察

本邦での狂犬病曝露前免疫は，組織培養不活化ワクチン1回量1.0 mLを4週間隔で2回，その後6～12カ月後に1回皮下注射する方式が標準である[8]．しかし，今回生じたようなワクチン供給不足の状況において，1人に1回量1.0 mLを投与すれば，短期間にワクチンが枯渇して，実際上狂犬病ワクチン接種が不可能となるであろう．高山らは，狂犬病ワクチン成分にアレルギー反応がある患者に皮内接種で曝露前免疫を行った例を報告している[7]．本報告では，患者に全身反応はなく，有意な抗体上昇がみられ，国産ワクチンを用いても皮内接種による曝露前免疫が可能であることが示唆された．

今回われわれは，ワクチン不足の状況下でも，できる限り多くの人々からの要望に応えられるように，狂犬病ワクチンの1回接種量を減量しても効果が得られる方法として，タイ赤十字方式を改変した皮内接種法(駒込方式)を考案し，その有効性と安全性を検討した．小規模な接種試験であったが，接種者全員の抗体価が0.5 EU/mL 以上となった．これはWHOの定める発症防御レベルの0.5 IU/mL以上と同等とみなすことができる．また，接種部位の発赤など軽度の局所副反応を認めたが，全身的副反応は認められなかった．

わが国における狂犬病ワクチンの生産量は，定期接種のワクチンに比べれば非常に少なく，急激な需要の増大が起これば，狂犬病ワクチンが不足する事態は避けられない．ワクチン不足への対処法として，ワクチンの備蓄や外国から緊急輸入などの方策が考えられる．しかし，ワクチンの備蓄は期限切れで廃棄することになる可能性や，外国産のワクチンの輸入に関しては法的な制限もあり，現実的には難しい．また，ワクチンは急に増産することができない．交通手段の発達により，日本から数時間以内で狂犬病常在地に渡航できることを考えると，今後も輸入狂犬病患者が発生し，再び狂犬病ワクチンの供給不足が発生する可能性があることを十分認識する必要がある．

今回検討した駒込方式は，接種量が少なくとも，標準法に劣らず効果がみられ，かつ副反応も軽微であるため，ワクチン不足時にはもちろん，平時にも使用できる有効な曝露前免疫法となる可能性があり，今後はより多くの被接種者を対象として，さらなる検討を行う価値がある．

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Pre-Exposure Prophylaxis for Rabies with Intradermal Injection Using Japanese Rabies Vaccine

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In November 2006, two consecutive imported rabies cases were reported in Japan. The demand for rabies vaccine has grown rapidly, resulting in a shortage of the vaccine. Therefore, pre-exposure prophylaxis for rabies was restricted. In order to prepare for the vaccine shortage, it is necessary to consider a method that is effective yet uses less amount of the vaccine. The intradermal method we tested uses only 20% of the vaccine dose required under the standard method, but every subject tested had a sufficient rise in their anti-rabies antibody titer. This was a small inoculation trial, but intradermal vaccination is an effective method, and may be used on a regular basis not only when vaccine is short.

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Migration behaviour and pathogenesis of five ascarid nematode species in the Mongolian gerbil *Meriones unguiculatus*

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Abstract

To understand the characteristic features of the Mongolian gerbil, *Meriones unguiculatus*, as an animal model of ascarid infections, the migration behaviour and pathogenesis of larvae were investigated in experimentally infected gerbils. Embryonated eggs from each of *Toxocara canis*, *Baylisascaris procyonis*, *B. transfuga*, *Ascaris suum*, and *A. lumbricoides* were orally inoculated into gerbils and larvae were recovered from various organs at designated periods. In *T. canis*-infected gerbils, larvae were present in the liver 3 days after infection and in the skeletal muscle and brain via the heart and lungs at a similar rate. In *B. procyonis*- and *B. transfuga*-infected gerbils, larvae were present in the lungs within 24 h after infection, with some having reached the brain by that time. After 24 h, larvae of *B. procyonis* tended to accumulate in the brain, while those of *B. transfuga* accumulated in skeletal muscles. In *A. suum*- and *A. lumbricoides*-infected gerbils, larvae remained in the liver on day 5 post-infection and elicited pulmonary haemorrhagic lesions, which disappeared 7 days after initial infection. Thereafter, no larvae of any type were recovered. Ocular manifestations were frequently observed in *T. canis*- and *B. procyonis* infected gerbils, but were rare in *B. transfuga*-infected gerbils. In the cases of *A. suum* and *A. lumbricoides*, migration to the central nervous system and eyes was extremely rare, and larvae had disappeared by 2 weeks post-infection. Fatal neurological disturbances were observed in *B. procyonis*-infected gerbils, whereas irreversible non-fatal neurological symptoms were observed in the case of *B. transfuga*.

Introduction

Larval stages of ascarid nematodes elicit severe tissue damage when they invade hosts which are not normally the definitive host. The racoon roundworm, *Baylisascaris procyonis*, is particularly prone to cause a fatal neurological disturbance (Huff *et al.*, 1984; Kuchle *et al.*, 1993; Moertel *et al.*, 2001; Wise *et al.*, 2005). The dog roundworm, *Toxocara canis*, and the cat roundworm, *T. cati*, are also responsible for the visceral larva migrans syndrome (VLM) in humans (Glickman & Magnaval, 1993; Fisher, 2003). The VLM caused by these two roundworms is commonly known as toxocariasis, which is considered a disease of infants and children, although adults are also infected (Glickman *et al.*, 1987; Aragane *et al.*, 1999; Yoshida *et al.*, 1999). Moreover, outbreaks of VLM due to the pig roundworm, *Ascaris suum*, have been reported from the southern part of Japan (Maruyama *et al.*, 1996; Sakakibara *et al.*, 2002). Meningitis can occur when *Ascaris* larvae migrate into the central nervous system

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(Osoegawa *et al.*, 2001). However, a precise diagnosis of these animal-borne ascarid infections is not always possible and an adequate anthelminthic therapy against these infections was not available. The lack of a comprehensive study using animal models for ascarid infections has also prevented progress in this field.

The Mongolian gerbil *Meriones unguiculatus* is known to be susceptible to a variety of parasites including *Brugia pahangi* (Ash & Riley, 1970), *Strongyloides stercoralis* (Nolan *et al.*, 1993), *Nippostrongylus brasiliensis* (Horii *et al.*, 1993), and *Entamoeba histolytica* (Chadee & Meerovitch, 1984). Akao *et al.* (2000) and Takayanagi *et al.* (1999) demonstrated that gerbils could serve as an animal model for ocular toxocariasis due to both *T. canis* and *T. cati*. The occurrence of retinal haemorrhages including larval invasion into the retina was found to be quite high compared with that in mice after oral inoculation of infective eggs. However, no information is available on the migration route, the final site of infection, or on the pathogenesis of ascarid larvae in gerbils. Here, we present our findings on the characteristic features of ascarid infections in gerbils as they relate to the pathogenesis of VLM in humans.

Materials and methods

Mongolian gerbils *Meriones unguiculatus* ranging between 2 and 3 months of age, were raised in the Animal Centre of Nippon Medical University and were maintained under pathogen-free conditions. Only male gerbils with black hair were used and all experiments were carried in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Recovery of ascarid eggs

Eggs of *T. canis* were obtained from the uteri of adult worms collected from faeces following the administration of anthelmintics to naturally infected puppies. Worms of *B. procyonis* were recovered from the intestine of infected racoons (Sato *et al.*, 2002), and those of *B. transfuga* collected from the faeces of infected bears. Worms of *B. transfuga* were naturally expelled from bears during their fasting period just prior to hibernation. Adult worms of *A. suum* were obtained from slaughtered pigs and *A. lumbricoides* were collected after treatment with anthelmintics from naturally infected humans. Female worms of each species were isolated, and matured embryonated eggs were prepared following the method of Oshima (1961).

Fully embryonated eggs were treated with 50% hypochlorous acid for 10 min to remove their proteinous membranes. After repetitive washing with distilled water, 1000 eggs of each ascarid were inoculated into each gerbil through a gastric tube under light anaesthesia.

Recovery of larvae

After ophthalmological observations (Takayanagi *et al.*, 1999), four gerbils from each group were sacrificed using sodium pentobarbital at predetermined intervals. One gerbil from each group was examined histopathologically and the remainder were used for larval recovery from the gastrointestinal tract, liver, lungs with heart, and skeletal muscle including bone and genital organs except for the skin. The contents of the gastrointestinal tract were collected in a conical tube at 6 and 12 h after inoculation. Each organ was minced and digested with artificial gastric juice (0.5% 1:10,000 pepsin, 0.7% hydrochloric acid) for 2 h at 37°C along with vigorous agitation. After digestion, the fluids were sieved with a tea strainer, and centrifuged at 320 × g for 5 min. The supernatant was discarded and a small amount of distilled water was added to the tube. The sediment was then spread out on glass, and larvae in the fluid were counted using a stereoscopic microscope. Examination of the brain tissue was performed as follows: each brain including the olfactory bulb, cerebrum, cerebellum, and pons was enucleated and minced into small pieces (approximately 2 mm^3) on a slide glass using forceps, and these were then covered with another slide glass. Migrating larvae were then counted using a microscope with eight or nine slide glasses being examined in each sample. Recovery rates were calculated from a mean of three gerbils at each period.

Histopathology

Tissue samples of liver, lungs, femoral muscle, brain, and gastrointestinal tract including the stomach, duodenum, ileum, caecum and rectum were fixed in 10% neutral formalin solution. Serial sections were prepared and stained with haematoxylin and eosin or periodic acid Schiff haematoxylin.

Results

Changes in the recovery rates of larvae from various organs after oral inoculation of the five ascarid species were recorded (fig. 1) and recovery rates arranged in the order of the migration route shown in fig. 2. In *Toxocara canis*-infected gerbils, almost all larvae were recovered from the intestinal wall up to 24 h after infection. Thereafter, larvae began to appear in the liver and lungs (by day 3), and then in the skeletal muscle and brain. The number of larvae in the muscle and brain were approximately equal. Macroscopically, haemorrhagic lesions which were observed in the lung 3, 5, 7 and 14 days after infection gradually disappeared. Ophthalmoscopically, a motile larva was observed in the retina 14 days after infection.

In *B. procyonis*- and *B. transfuga*-infected gerbils, the recovery rates of larvae were significantly lower than that in the case of *T. canis*. Both species of larvae had migrated into the lungs through the liver within 24 h after infection, and some had already arrived in the brain by this time, resulting in small haemorrhagic foci in the brain (fig. 3). With *B. procyonis*, ocular invasion by the larvae was observed and neurological disorders such as rotational and involuntary movement or paraplegia emerged, resulting in the mortality of gerbils between days 15 and 20 post-infection. Macroscopically, the gerbils exhibited fresh petechial haemorrhages of the lung 1 day after infection, but no new haemorrhagic lesions were evident beyond that time. Ophthalmic examination

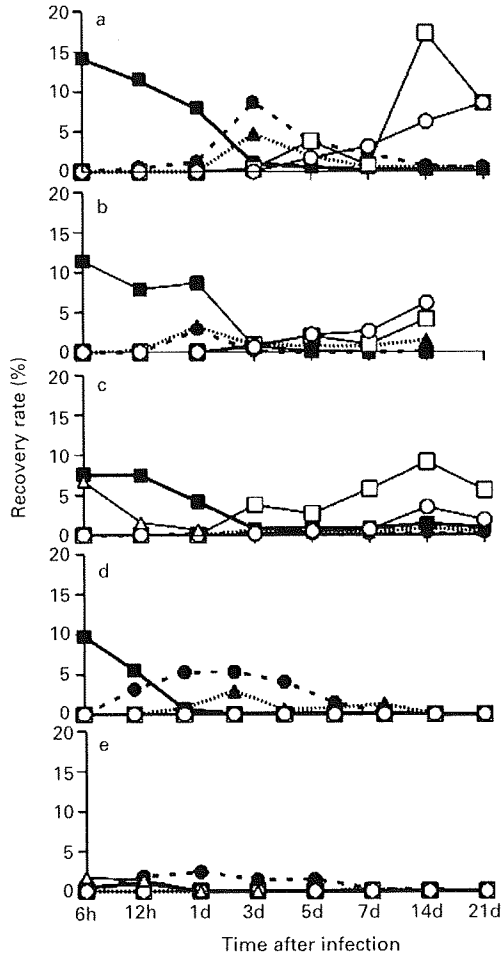

Fig. 1. Recovery rates (%) of larvae from various organs in gerbils after oral inoculation with five ascarid species up to day 21 post-infection. a, *Toxocara canis*; b, *Baylisascaris procyonis*; c, *B. transfuga*; d, *Ascaris suum*; e, *A. lumbricoides*; ■, gastrointestinal tract; ●, liver; ▲, lung and heart; □, muscles; ○, brain; △, intestinal contents.

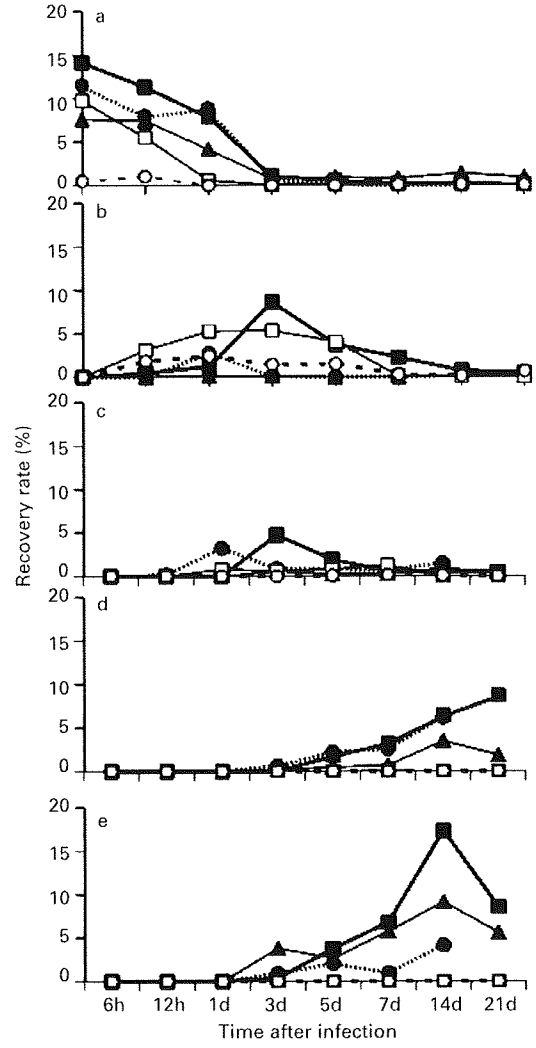

Fig. 2. Recovery rates (%) of larvae in gerbils after oral inoculation with five ascarid species to show the route of migration. ■, *Toxocara canis*; ●, *Baylisascaris procyonis*; ▲, *B. transfuga*; □, *Ascaris suum*; ○, *A. lumbricoides*; a, gastrointestinal tract; b, liver; c, lung and heart; d, brain; e, muscles.

demonstrated motile larvae in the retina and severe chorioretinitis 7 days after infection. The number of migrating larvae of *B. procyonis* in the brain was higher than that of *B. transfuga*-infected gerbils at all times after the infection. By day 3 post-infection an average of 6.3 larvae (range 6–7) in *B. procyonis*-infected gerbils and 1.7 larvae (range 1–2) in *B. transfuga*-infected gerbils were found. By day 7, an average of 26.0 larvae (range 23–31) in *B. procyonis*-infected gerbils and 6.7 larvae (range 5–8) in *B. transfuga*-infected gerbils were recovered. Ophthalmic and neurological abnormalities were less severe than those observed with *B. procyonis*, although gait difficulty and circulatory movements in the same direction were presented by day 10 post-infection. The number of *B. transfuga* larvae in the skeletal muscle was higher than that in the brain; i.e. an average number of larvae recovered were 37.7 (range 21–67) in muscle and 1.7 (range 1–2) in brain at the day 3 post-infection, and 91.0 (range 95–100) in muscle and 34.7 (range 24–49) in brain at the day 14 post-infection.

With *A. lumbricoides*, the average recovery rate of larvae after 6 h of infection was 2.3% (range 1.7–2.6%) compared with about 10% or higher in other ascarid parasites (14.1% in *T. canis*, 11.4% in *B. procyonis*, 16.3% in *B. transfuga* and 9.7% in *A. suum*; fig. 1a). Migrating larvae were present in the liver 12 h after infection, and remained there for up to 5 days after infection in the case of *A. lumbricoides* and