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Parasitology International 57 (2008) 495–498

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

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

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ARTICLE INFO

Article history:
Received 17 January 2008
Received in revised form 19 June 2008
Accepted 27 June 2008
Available online 9 July 2008

Keywords:
Toxocara canis
Prolactin
Lactational transmission

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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doi:10.1016/j.parint.2008.06.006

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 ($P<0.05$).

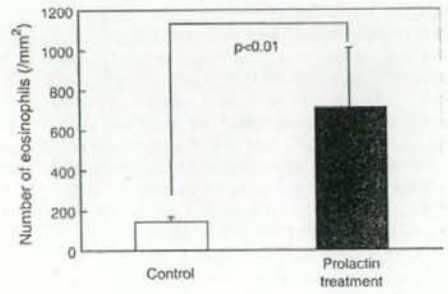

Fig. 1. Eosinophil counts around the capsules of mammary glands in mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 713.6±293.6 cells/mm² in the prolactin-treated group, and 144±21.3 cells/mm² in the saline-treated 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.

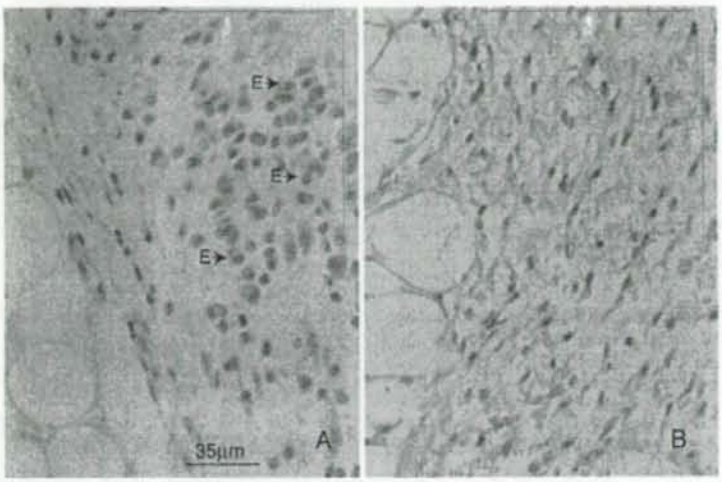

Fig. 2. Histopathological findings of mammary glands around the connective tissue in mice. Serial sections of mammary glands of female mice were stained with haematoxylin and eosin. Markedly higher eosinophilic (E) infiltrations around the connective tissue of the mammary gland were observed in the prolactin-treated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

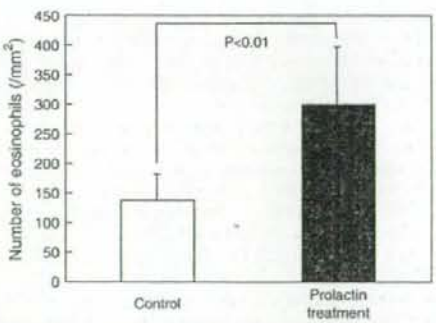

Fig. 3. Eosinophil counts around the capsules of mammary glands in chronically infected mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 300.8±95.6 cells/mm^2 in the prolactin-treated group, and 137.6±44.1 cells/mm^2 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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RESEARCH NOTES

J. Parasitol., 94(5), 2008, pp. 000–000

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.

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)
(DOI: 10.2169/internalmedicine.47.0626)

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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Received for publication September 20, 2007; Accepted for publication November 5, 2007
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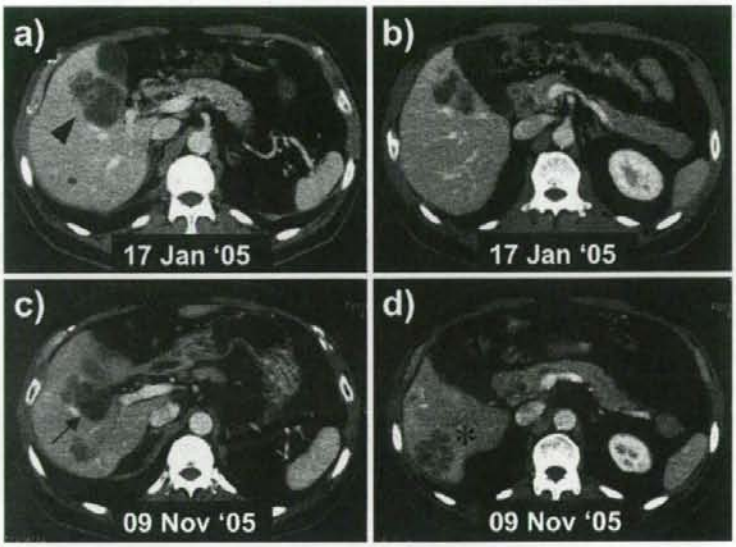

Figure 1. **The contrast-enhanced CT image presented huge cystic and multilocular lesions. a, b) In the anterior segment of the right lobe, a huge and low-attenuated mass (arrowhead) and some tiny hypodense lesions were detected. c) The corresponding lesion in the anterior segment migrated into the center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

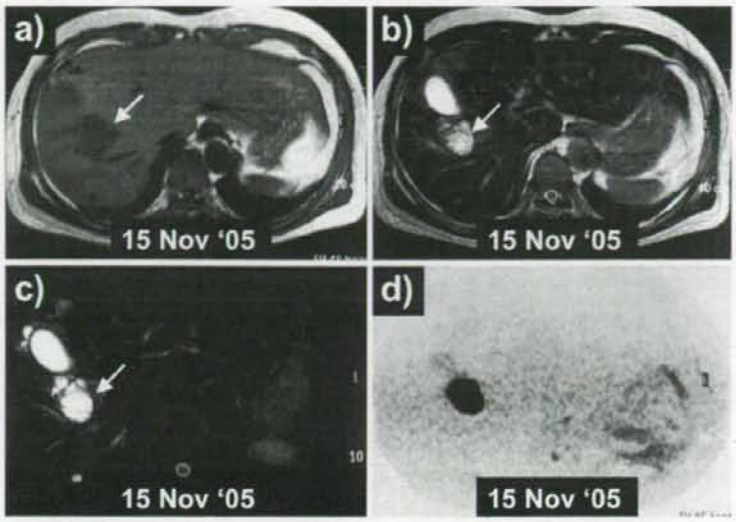

Figure 2. **The corresponding MR image showed necrotic or abscess-forming lesions (arrows); a) T1-weighted images, b) T2-weighted images, c) fat-suppressed T2-weighted image, d) inverted diffusion-weighted images.**

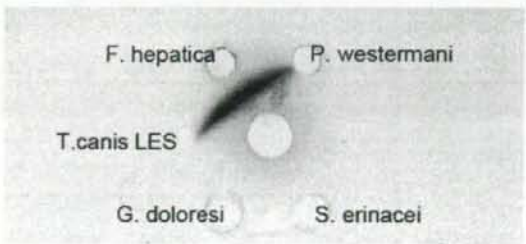

Figure 3. **The ouchterlony double-diffusion test showed a strong precipitin band against crude antigen on *Fasciola hepatica*. The positions of antigens; F. hepatica, *Fasciola hepatica*; P. westermani, *Paragonimus westermani*; T. canis LES. Larval excretory and secretory antigen of *Toxocara canis*: 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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Parasitology International 57 (2008) 525–529

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Case report

A familial case of visceral toxocariasis due to consumption of raw bovine liver

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ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 4 August 2008
Accepted 6 August 2008
Available online 16 August 2008

Keywords:
Visceral toxocariasis
Transmission route
Paratenic host
Raw liver
Sashimi
Sonazoid-contrast US

ABSTRACT

We present 3 adult cases of visceral toxocariasis from the same family, who each consumed thin slices of raw bovine liver weekly, and developed eosinophilia and multiple small lesions in their livers and lungs. Serological examinations using the larval excretory–secretory product of *Toxocara canis* strongly indicated infection with *Toxocara* species larvae. The patients responded well to treatment with albendazole. Ingestion of raw liver from paratenic animals is considered to be a common transmission route of human toxocariasis, especially in adults.

1. Introduction

Human toxocariasis is a common helminthozoonosis caused by infestation with larvae of the nematode worms *Toxocara* (*T.*) *canis* or *T. cati* [1–5]. It has long been considered a parasitic disease that affects pet owners and children, because transmission was thought to only occur via ingestion of infective embryonated eggs after exposure to soil and hair contaminated with the feces of dogs and cats. However, infective stage larvae can also be transferred to other animals and humans through predation, and this type of parasite transfer is now considered to be frequently related to adult cases of toxocariasis in Japan [6]. Therefore, toxocariasis should be recognized as a food-borne parasitic disease, especially in societies where consumption of raw meat is prevalent. Herein, we present 3 adult cases of visceral toxocariasis from the same family who regularly consumed thin slices of bovine liver. Our findings show that consumption of raw liver from paratenic animals is an important source of infestation.

2. Cases

A 58-year-old man (Patient 1) had never been found with leukocytosis in annual medical check-up examinations until December, 2007, when an increased number of white blood cells (11,800/μl) with marked eosinophilia, absolute count 4250/μl, and elevated IgE (2345 U/ml, normal <100) were found. He was referred to Nara Medical University Hospital. At the initial interview, the patient noted that he and 2 other family members, his 57-year-old wife (Patient 2) and 27-year-old son (Patient 3), consumed raw bovine liver every Friday for the past year, believing that it was good for their health. Their habit was to obtain 100 g of raw bovine liver at a nearby meat shop and serve it as thin slices at dinner. Patient 1 generally consumed the most, followed in order by Patient 2 and Patient 3. In contrast, the mother of Patient 1, who lived in the same house, only ate the raw liver on a few occasions.

We performed blood examinations for all 4 family members. Although none was symptomatic, the 3 regular consumers showed increased eosinophils and IgE (Table 1), while the mother who consumed raw liver only rarely showed no eosinophilia or elevated IgE. Results of a blood examination for Patient 2 obtained by a local physician 1 year previously, prior to beginning the dietary habit, showed a normal number of white blood cells at 6000/μl with a 3%

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1383-5769/$ – see front matter
doi:10.1016/j.parint.2008.08.002

Table 1
Patient laboratory data

	Patient 1	Patient 2	Patient 3
WBC (/μl)	11,800	8700	8800
Eo (%)	36.0	27.0	19.3
Hb (g/dl)	14.7	14.5	15.6
PLT ($\times 10^4$/μl)	28.4	25.4	29.0
CRP (mg/dl)	0.2	0.5	0.1
AST (IU/l)	23	24	17
ALT (IU/l)	12	28	22
ALP (IU/l)	279	227	201
γGTP (IU/l)	35	53	21
IgE (U/ml)	2345	645	422

Abbreviations and normal ranges:
WBC: white blood cells, 3900–9800/μl.
Eo: eosinophils, 0–5%.
Hb: hemoglobin, 13.2–15.6 g/dl.
PLT: platelet, 13.0–36.0 ($\times 10^4$)/μl.
CRP: C-reactive protein, less than 0.2 mg/dl.
AST: aspartate aminotransferase, 12–32 IU/l.
ALT: alanine aminotransferase, 5–36 IU/l.
ALP: alkaline phosphatase, 120–360 IU/l.
γGTP: gamma-glutamyl transpeptidase, 11–69 IU/l.
IgE: immunoglobulin E, less than 100 U/ml.

eosinophil fraction, though IgE was not examined. Additional tests were performed to determine the etiology of the hypereosinophilia in the patients. Chest computed tomography (CT) demonstrated multiple small pulmonary lesions, nodules with halos and poorly defined margins, and ground-glass opacity with a poorly defined margin in all. Furthermore, contrasted abdominal CT in the portal phase revealed multiple, poorly defined, low-attenuated nodules in the liver of Patient 1, while Patients 2 and 3 each had only a single lesion. Representative CT images from Patients 1, 2, and 3 were shown in Fig. 1. Some nodules in the liver of Patient 1 showed peripheral rim enhancement in the arterial phase (Fig. 2A), while most nodules were undetectable in the equilibrium phase. These CT findings of pulmonary and hepatic lesions were very consistent with those in previous reports of toxocariasis [7–9]. Ultrasonography (US) detected multiple, small, oval, hypoechoic lesions in the liver of Patient 1, and 3 hypoechoic lesions in the liver of Patient 2 including the one lesion detected by CT, whereas none was detected in Patient 3. We also performed contrast US using a newly developed material, Sonazoid® [10], and compared those images with the CT images (Fig. 2B). The lesions were detected as hypoechoic areas in the portal phase and even more clearly in the equilibrium phase, while they were not enhanced in the arterial phase, suggesting that the lesions were poorly supplied with arterial or portal blood. In the post-vascular or so-called Kupffer image phase, the lesions remained un-enhanced, suggesting the absence or scant presence of Kupffer cells (Fig. 2C).

A rapid diagnostic test for toxocariasis, ToxocaraCHECK® [11], which detects IgG antibodies against the larval excretory–secretory (LES) product of *T. canis* on an antigen-sensitized nitrocellulose membrane, showed positive results for all 3 patients. Furthermore, a microplate enzyme-linked immunoabsorbent assay (ELISA) using the LES product and serum from each patient diluted 1:900 revealed the presence of human IgG antibodies at very high titers. The optical density (OD) values at 405 nm for sera from Patients 1, 2, and 3 were 1.58, 1.41, and 1.38, respectively, as compared to the established OD value cutoff level of ≤0.2 for serum from healthy individuals. We also examined immunopositivity against nematode antigens other than the LES product of *T. canis* using a gel diffusion test (Fig. 3), which revealed a strong positivity against the LES products of both *T. canis* and *T. cati*, suggesting a high cross-immunogenicity between them or dual infection, though no formation of precipitate was observed against the LES product of *Ascaris suum* or *Anisakis simplex*. Since no serological examination has been established yet to discriminate between toxocariasis caused by *T. canis* and that by *T. cati*, we made a diagnosis of toxocariasis by *Toxocara species* for all 3 patients.

The patients were instructed regarding prevention of re-infection and treated with a 4-week regimen of daily albendazole at 600 mg (10.8 mg/kg of body weight for Patient 1, 12.8 mg/kg for Patient 2, 10.0 mg/kg for Patient 3). All completed the treatment, though a mild elevation of transaminases up to double the upper limit was observed in Patient 2. During treatment, the eosinophil count decreased in each and became normalized by the end of treatment in Patient 2, while Patients 1 and 3 were further treated with albendazole at the same dose for two more weeks until the eosinophil count became normalized. Hepatic and pulmonary lesions were undetectable by CT and US examinations at the end of treatment in all of the patients. Three months after finishing the treatment with albendazole, we confirmed that a normal eosinophil count was maintained in each patient, along with no recurrent hepatic or pulmonary lesions in CT findings. In addition, the OD values of anti-*T. canis* LES were decreased to 0.95, 0.80, and 0.74 from the initial values of 1.58, 1.41, and 1.38 before treatment in Patients 1, 2, and 3, respectively.

3. Discussion

Visceral toxocariasis is a representative infection of visceral larva migrans (VLM), first reported by Beaver et al. [12], known to be prevalent among preschool children, as they tend to play with dogs in open areas and ingest egg-contaminated soil. However, a recent review of human toxocariasis cases in Japan noted that the disease affects predominantly adults rather than children [6].

There are a number of case reports of adult toxocariasis [13–21], and accumulating evidence [22–27] has revealed that a common route of adult human infection is through ingestion of uncooked or raw liver from a paratenic host. In general, transfer of infective stage larvae through predation is a common mode of helminth transmission among carnivorous vertebrates and this type of parasite transfer can also occur from animals to humans. In experiments with chicken, cattle, and swine, Taira et al. found that the animals were able to function as paratenic hosts for *T. canis* and that the liver was one of the most intensely affected organs [22,23]. Similar observations regarding the importance of predatory cycle have also been reported for cases of infection with *A. suum* [28,29].

Adults with a dietary habit of consuming raw liver have been found to be at high risk for human VLM [24–26]. Morimatsu recently reported an interesting familial case in Japan, in which a father (71 years old) and son (45 years old) developed visceral toxocariasis after consumption of raw chicken livers, and found *T. canis larvae* in the livers of chickens raised in their breeding farm [17]. The present patients began to eat raw bovine liver weekly and continued the habit for about 1 year. Patients 1 and 2 had normal white blood cell counts including eosinophils in routine peripheral blood examinations conducted 1 year and just prior, respectively, to beginning the weekly consumption of raw bovine liver, which suggests that the dietary habit of eating raw liver contributed to toxocariasis in those cases. We strongly suspect that some of the raw liver served at dinner was infected with larvae of *Toxocara species*. Thus, it is important to recognize that toxocariasis can be a food-borne parasitic disease, based on the present findings.

The majority of patients with visceral toxocariasis are asymptomatic and the disease is often discovered during investigation of peripheral eosinophilia [8,9,30], as in the present cases, though those with a high number of worms may complain of vague abdominal discomfort, abdominal pain, cough, dyspnea, fever, or general weakness. Although each of our patients were asymptomatic, the degree of eosinophilia, serum IgE level, and number of hepatic lesions were prominently high in Patient 1, who ingested larger quantities of raw liver as compared to the others, suggesting that the number of worms and disease severity may be proportional to the amount of raw liver intake.

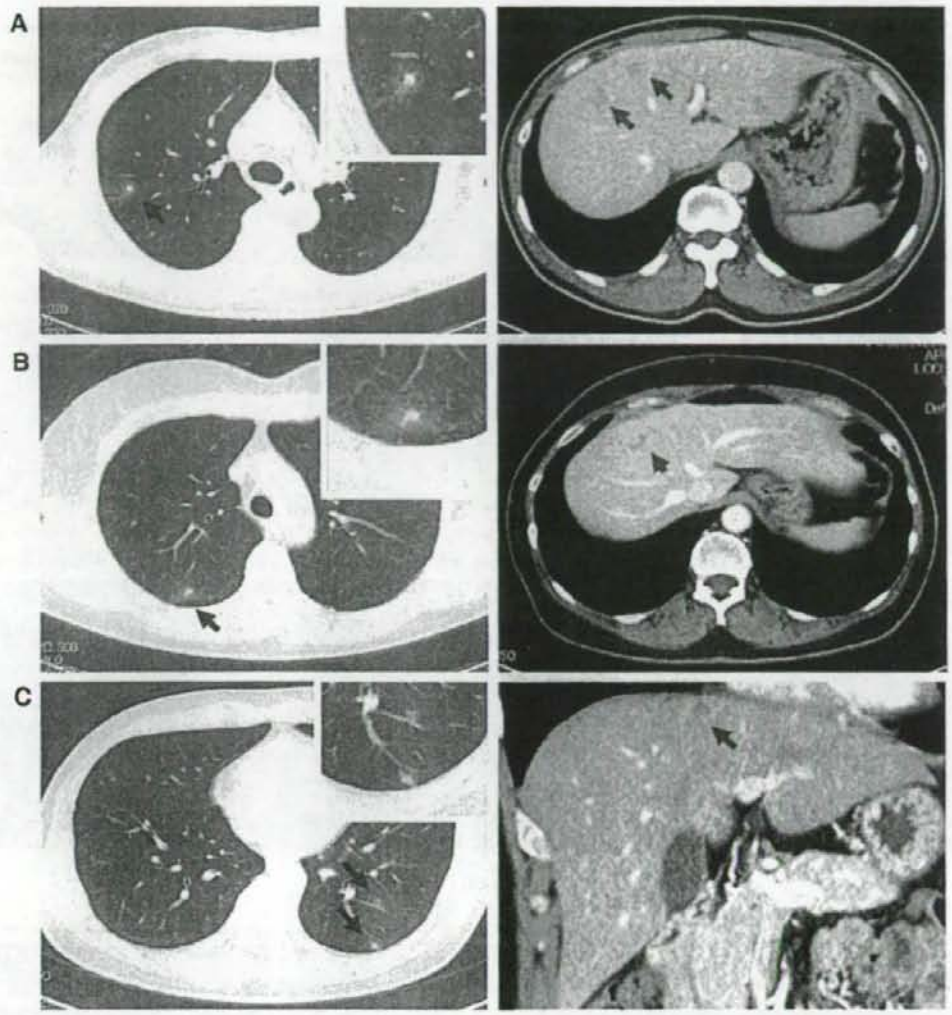

Fig. 1. CT images of pulmonary and hepatic lesions. A, B, and C show representative CT images from Patients 1, 2, and 3, respectively. Pulmonary lesions (arrows, magnified in inset) were shown as nodules with halos and a poorly defined margin or ground-glass opacity with a poorly defined margin. Hepatic lesions (arrows) appeared as small, poorly defined areas of low-attenuation in the portal venous phase of contrast-enhanced CT scanning.

We found that imaging modalities were very useful to reach a diagnosis. Characteristic CT findings of hepatic and pulmonary lesions in visceral toxocariasis reported elsewhere [7–9] are compatible to those found in our patients. Typically, the hepatic lesions are multiple, small (usually less than 2 cm in diameter), poorly defined, oval or elongated, and with low attenuation, and usually best visualized in the portal venous phase of contrast CT. Pulmonary lesions are shown as multiple small nodules (mostly less than 3 cm in diameter), with some associated with halos with poorly defined margins, and also shown as ground-glass opacity with a poorly or well-defined margin. Lesions in the liver and lung tend to be found in the periphery of those organs. In the present cases, we also performed US examinations using Sonazoid, a recently developed microbubble contrast agent, which is phagocytosed by liver-specific macrophages, known as Kupffer cells, following the vascular phase [10]. Sonazoid-contrast US showed that the liver lesions were poorly supplied with arterial and portal blood, and contained no or few Kupffer cells as compared with the surrounding liver parenchyma. These CT and US image findings are compatible to inflammatory granuloma. Although we did not perform a puncture biopsy of the hepatic lesions for histological examinations, eosinophilic granuloma would be expected.

A serological examination is also important for an accurate diagnosis, as it is difficult to obtain worms from patients in most cases. In the present cases, we performed 3 kinds of serological tests, rapid screening ELISA, quantitative ELISA, and an immunodiffusion test, using the LES product of *T. canis*, which is known to be highly immunogenic. Sera from the 3 patients were positive in all of those tests. However, in immunodiffusion tests with LES products of *T. canis*

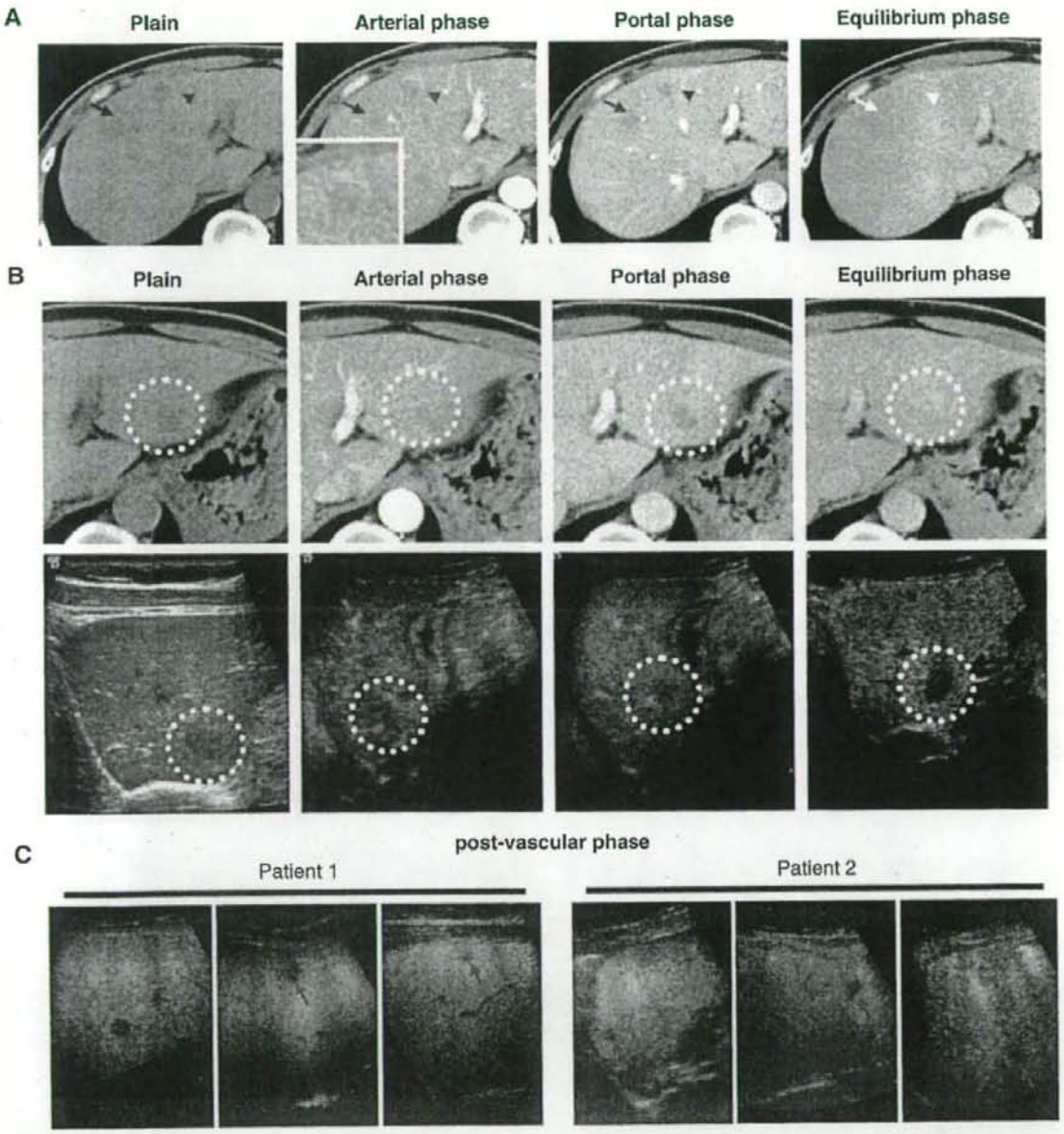

Fig. 2. CT and US images of hepatic lesions. A. Two nodules (arrow and arrowhead) in the liver of Patient 1 are shown. That shown by the arrowhead (magnified in inset) had weak peripheral rim enhancement in the arterial phase, while both were undetectable in the equilibrium phase. B. A lesion (circle with broken line) in the left lobe of Patient 1 was targeted with Sonazoid-contrast US (lower) and the results were compared with contrast CT images (upper). With CT, the lesion was best seen in the portal phase and became undetectable in the equilibrium phase, while it was clearly shown as an un-enhanced area in the equilibrium phase. C. Post-vascular Sonazoid-contrast US images revealed that the lesions (arrows) remained hypoechoic. Three lesions each from Patients 1 and 2 are shown.

and *T. cati*, sera from the patients were reactive to both of the LES products, because of their high cross-immunogeneicity. Finally, we made a diagnosis of toxocariasis by *Toxocara species*.

Covert toxocariasis with eosinophilia alone is often treated conservatively after instruction regarding prevention of re-infection. Stopping the habit of ingesting raw liver alone might have been adequate for the present cases of asymptomatic toxocariasis. However, the existence of living larvae in the lungs and liver for a prolonged period is a potential risk for their migration to other organs, including the spinal cord and brain, leading to serious complications. We decided to prescribe albendazole, which is commonly used for toxocariasis and known to be effective with minimal adverse reactions. A dose of

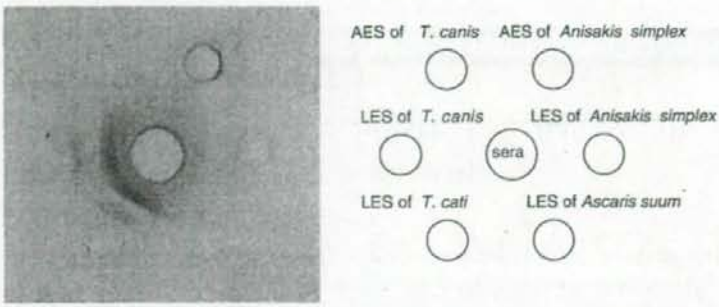

Fig. 3. Results of serological tests. Data from Patient 1 shown with schematic positions of antigens and sera are presented as representative findings. The antigens used were adult worm extract (AES) of *T. canis*, AES of *Anisakis simplex*, larval excretory–secretory (LES) of *Anisakis simplex*, LES of *Ascaris suum*, LES of *T. cati*, and LES of *T. canis*. Strong precipitin bands were observed for the LES products of *T. canis T. cati* in serum samples from all 3 patients.

400 mg of albendazole twice a day or 10 mg/kg of body weight/day in two divided doses for 5 days seems to be the currently recommended therapy [4,31,32], though the optimal duration of therapy is unknown [33]. According to a previous report [31], only 32% of patients with toxocariasis were clinically cured with a 5-day regimen and other reports have noted that additional treatments with other anthelminthic drugs, such as diethylcarbamazine and mebendazole, or the use of albendazole for a longer period was effective [18,34–39]. We adopted a 4-week regimen of daily albendazole at 600 mg, with the disappearance of eosinophilia considered to mark the endpoint of therapy. Clinical improvement appeared soon after the initiation of treatment, demonstrated by a decrease in eosinophil count, with minimum adverse effects related to mild liver dysfunction, followed by the disappearance of hepatic and pulmonary lesions.

Based on our results, we concluded that infestation with *Toxocara species* from paratenic animals is likely a common and important mode of transmission to humans, especially adults, in areas such as eastern Asia where the consumption of raw liver remains a cultural habit.

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INTERNAL MEDICINE

□ PICTURES IN CLINICAL MEDICINE □

Visceral Toxocariasis from Regular Consumption of Raw Cow Liver

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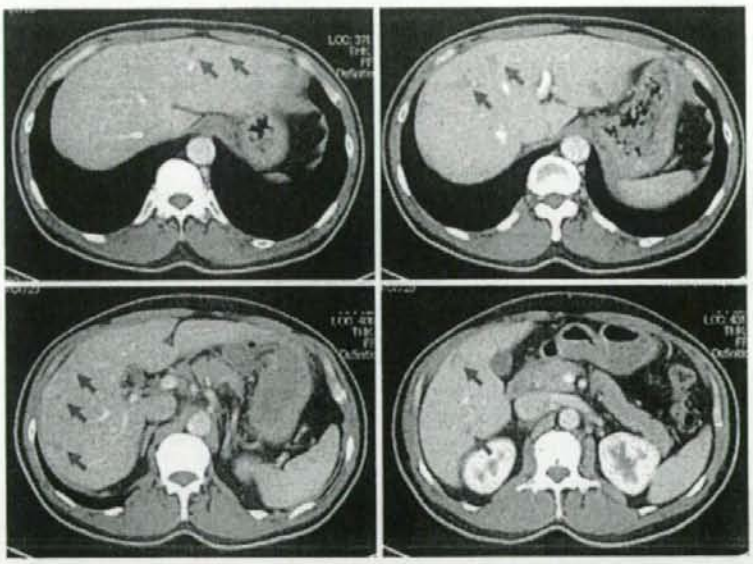
Key words: toxocariasis, visceral larva migrans (VLM), albendazole, mode of transmission, raw liver

(Inter Med 47: 1289-1290, 2008)
(DOI: 10.2169/internalmedicine.47.1191)



Picture 1. 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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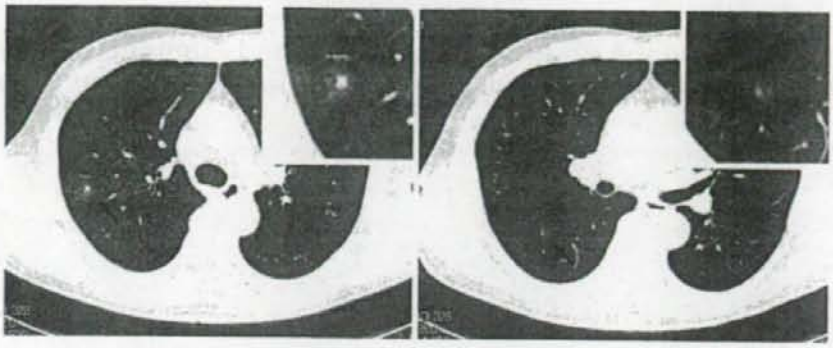

Picture 2. Chest CT scan image showing nodule with halo and ill-defined margin (left), and groundglass opacity with an ill-defined margin (right). The lesion is shown magnified in the inset.

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.

Research

Exposure to Bisphenol A Prenatally or in Adulthood Promotes T_H2 Cytokine Production Associated with Reduction of $CD4^+CD25^+$ Regulatory T Cells

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Background: Bisphenol A (BPA) is a widespread endocrine-disrupting chemical that can affect humans and animals.

Objectives: We investigated the effects of adult or prenatal exposure to BPA on T-helper (T_H)1/T_H2 immune responses and the mechanisms underlying these effects.

Methods: To evaluate the effects of exposure to BPA in adulthood, male *Leishmania major*–susceptible BALB/c and –resistant C57BL/6 mice were subcutaneously injected with 0.625, 1.25, 2.5, and 5 μmol BPA 1 week before being infected with *L. major*. To evaluate prenatal exposure, female mice were given BPA-containing drinking water at concentrations of 1, 10, and 100 nM for 2 weeks, then mated, and given BPA for another week. Male 10-week-old offspring were infected with *L. major*. Footpad swelling was assessed as a measure of the course of infection.

Results: Mice exposed to BPA prenatally or in adulthood showed a dose-dependent increase in footpad swelling after being infected with *L. major*. Exposure to BPA in adulthood significantly promoted antigen-stimulated production of interleukin (IL)-4, IL-10, and IL-13 but not interferon-γ (IFN-γ). However, mice prenatally exposed to BPA showed increased production of not only IL-4 but also IFN-γ. The percentages of $CD4^+CD25^+$ cells were decreased in mice exposed to BPA either prenatally or in adulthood. Effects of prenatal BPA exposure were far more pronounced than effects of exposure in adulthood.

Conclusion: BPA promotes the development of T_H2 cells in adulthood and both T_H1 and T_H2 cells in prenatal stages by reducing the number of regulatory T cells.

Key words: bisphenol A, cytokine, endocrine-disrupting chemicals, prenatal exposure, regulatory T-cells. *Environ Health Perspect* 116:514–519 (2008). doi:10.1289/ehp.10829 available via *http://dx.doi.org/* [Online 29 January 2008]

Bisphenol A (BPA), an estrogenic endocrine-disrupting chemical (EDC), has drawn attention because of its potential for human exposure. BPA is widely used, including in dentistry, food packaging, and lacquers to coat food cans and water pipes. It can enter the environment, resulting in chronic exposure of humans and wildlife. In fact, BPA has been detected not only in food and water (Heemken et al. 2001; Shin et al. 2001; Thomson and Grounds 2005) but also in human urine and blood samples as well as in the placenta and amniotic fluid of pregnant women (Ikezuki et al. 2002; Matsumoto et al. 2003; Schonfelder et al. 2002; vom Saal and Hughes 2005). BPA is one of the most widespread EDCs.

There is much evidence that exposure to BPA through contamination of the environment or the treatment of experimental animals disrupts developmental programs to alter sexual phenotypes and reproductive functions (Farabollini et al. 2002; Herath et al. 2004). BPA antagonizes the actions of thyroid hormone (Moriyama et al. 2002). Exposure of pregnant rats to BPA resulted in the chemical's transplacental transfer to the fetuses (Takahashi and Oishi 2000; Welshons et al. 2006), suggesting that developing embryos or fetuses might be affected by BPA. Prenatal exposure to BPA has been shown to alter a variety of reproductive endocrine parameters, such as testosterone and luteinizing hormone levels in rats (Ramos et al. 2003; Rubin et al. 2001) and the early onset of sexual maturation of female mice (Honma et al. 2002). In addition, behavioral changes have been reported in offspring of mice exposed to BPA during pregnancy and lactation (Dessi-Fulgheri et al. 2002). BPA may also be a potentially important modulator of immune responses. It inhibits adhesion capacity and promotes cytokine production in macrophages *in vitro* (Segura et al. 1999; Yamashita et al. 2005). Exposure to BPA also enhances the production of autoantibodies by B1 cells (Yurino et al. 2004). Furthermore, imbalanced T-helper (T_H)1/T_H2 immune responses have been demonstrated on exposure to BPA. BPA inhibits the secretion of interferon-γ (IFN-γ) in C57BL/6 and female NZB/NZW mice (Sawai et al. 2003). In contrast, BALB/c mice treated with BPA exhibit augmented T_H1 immune responses alone (Alizadeh et al. 2006), or both T_H1 and T_H2 responses (Yoshino et al. 2003). Our previous study indicated that BPA promotes T_H2 cytokine production *in vitro* and *in vivo* (Tian et al. 2003). However, the effects of prenatal exposure to BPA on immune responses have not been clarified.

In this study, we used mice infected cutaneously with *Leishmania major* to investigate the effect of BPA on T_H1/T_H2 immune responses in adulthood and prenatal stages. The model provides an excellent system with which to study the factors controlling the generation and regulation of T_H1 and T_H2 cells *in vivo*. Experimental infections of different strains of mice with *L. major* result in the development of either a predominant T_H1 response and resistance or a predominant T_H2 response and susceptibility. The early production of interleukin-12 (IL-12) and IFN-γ promotes a T_H1 response and healing, whereas IL-4 production is necessary for the development of a T_H2 response and of progressive disease. We also focused on $CD4^+CD25^+$ regulatory T cells (Treg cells), one of the $CD4^+$ T cell populations constitutively expressing the IL-2 receptor α-chain (CD25) playing a central and prominent role in the maintenance of the immunologic balance (Maloy and Powrie 2001; Shevach 2002) by inhibiting the proliferation of and the production of cytokines by $CD4^+$ and $CD8^+$ T cells (Dieckmann et al. 2005; Stassen et al. 2004). We evaluated whether $CD4^+CD25^+$ Treg cells were affected by exposure to BPA, resulting in the alteration of cytokine production by $CD4^+$ T cells.

Materials and Methods

Mice. Six- to 8-week-old *L. major*–susceptible BALB/c and *L. major*–resistant C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). Mice were housed in polymethylpentene (TPX) cages and fed sterile standard chow (FR-2; Funabashi Farm, Chiba, Japan). Drinking water was provided *ad libitum* in glass bottles. All animals were handled according to the guidelines of the Ethics Committee for Animal Experiments of Shinshu University. Animals were treated humanely and with regard for alleviation of suffering.

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This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas 14042222 from the Ministry of Education, Culture, Sports, Science and Technology.

The authors declare they have no competing financial interests.

Received 31 August 2007; accepted 25 January 2008.

Monoclonal antibodies and reagents. BPA was purchased from Nacalai Tesque (Kyoto, Japan). Phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)–conjugated anti-CD25 monoclonal antibodies (mAbs) were obtained from BD, Biosciences (San Diego, CA, USA). The cytometric bead array (CBA) kits were also from BD Biosciences.

Leishmania major. L. major (MHOM/SU/73/5ASKH) was kept in a virulent state by continuous passage in BALB/c mice. A cell suspension of popliteal lymph node from an infected BALB/c mouse was cultured in Schneider's medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% heat-inactivated fetal calf serum (FCS; Biocell Laboratories, Carson, CA, USA). Stationary phase promastigotes were collected by centrifugation and washed with saline. Mice were infected in the right hind footpad with 5×10^6 promastigotes. The course of infection was monitored by making weekly measurements of footpad thickness with a metric caliper. The results were expressed as the difference between the thickness of the infected right footpad and that of the noninfected left one.

To prepare soluble *L. major* antigen, 1×10^9 promastigotes were homogenized by three cycles of freezing and thawing in phosphate-buffered saline. Aliquots were stored at –30°C before use.

BPA treatment. **Exposure of adult male mice to BPA.** BPA was dissolved in corn oil and injected subcutaneously into the right hind leg at doses of 0.625, 1.25, 2.5 and 5 μmol, which is equivalent to 5.7, 11.4, 22.8, and 45.6 mg/kg body weight (bw). These doses were based on our previous study in which 1 μmol BPA was shown to increase IL-4 and IL-10 production in *Trichinella spiralis*-infected mice (Tian et al. 2003). The control mice received corn oil vehicle alone. One week later, the mice were injected with *L. major* promastigotes in the footpad of the same leg.

Prenatal exposure to BPA. Female mice were given BPA in drinking water at doses of 1, 10, and 100 nM for 2 weeks. Each group of mice was then mated with a male and treated with BPA-containing drinking water for another week. Offspring born within 16–19 days after BPA treatment was complete were used in this experiment. The 100 nM (about 3 μg/kg bw/day) dose of BPA was based on recent studies showing that administration of low doses of BPA at 2 and 20 μg/kg bw/day to pregnant animals caused permanent changes in reproductive organs of offspring (Honma et al. 2002; Nagel et al. 1997). The mice in all groups drank approximately 3–4 mL water per day. The total dose received by each female mouse during the period of experiment was about 0.07, 0.7, or 7 nmol. Offspring of dams who received drinking water without BPA were used as controls. Male 10-week-old offspring were infected with *L. major.*

In vitro ***culture of splenocytes.*** A single-cell suspension containing 2×10^6 splenocytes from each mouse was incubated in 24-well tissue-culture plates (Greiner, Nurtingen, Germany) in 1 mL RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS (Biocell Laboratories), penicillin (100 IU/mL), and streptomycin (100 μg/mL) (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were stimulated with *L. major* antigen (3 μg/mL) during the cultivation. Culture supernatants were collected 48 hr later and stored frozen until used.

Cytokine analysis. Concentrations of IL-4, IL-10, IL-13, and IFN-γ in culture supernatants were determined using CBA kits according to the manufacturer's instructions.

Flow cytometric analysis. Single-cell suspensions containing 1×10^6 splenocytes were stained with PE-conjugated anti-CD4 mAb and FITC-conjugated anti-CD25 mAb. The cells were washed, then analyzed using fluorescence-activated cell sorting (FACS) with a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software (BD Biosciences).

Statistical analysis. Results are presented as the mean ± SE. The statistical significance of the values was evaluated using Student's *t*-test. The significance was assessed at the $p < 0.05$ level of confidence.

Results

Effects of BPA on footpad swelling and cytokine production in **L. major–*infected adult male mice.*** Adult male mice injected with different doses of BPA were infected with promastigotes of *L. major* 1 week later. *L. major*–susceptible BALB/c mice developed a continuous increase in footpad thickness whether or not they were injected with BPA. The degree of swelling increased dose-dependently in mice treated with BPA. Mice exposed to 2.5 and 5 μmol of BPA developed significantly larger swelling than nonexposed control mice at weeks 6 and 8 after infection. Eight weeks after infection, footpad swelling was 1.49-fold greater in mice treated with 5 μmol of BPA than in controls (Figure 1A). However, infection with *L. major* among resistant C57BL/6 mice resulted in minimal swelling that began to resolve by 4 weeks after infection. There was no significant difference in footpad swelling among the groups (Figure 1B).

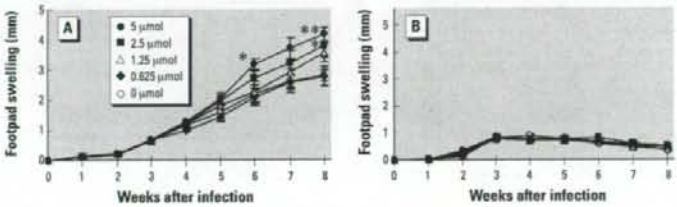

Figure 1. Effects of exposure to BPA in adult male BALB/c (*A*) and C57BL/6 (*B*) mice on footpad swelling after infection with *L. major.* Values represent mean ± SE ($n = 3$–4).
*$p < 0.05$ and **$p < 0.01$ compared with the nonexposed control group.

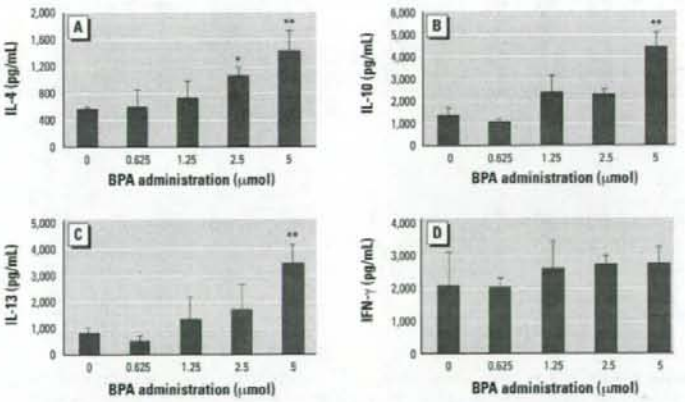

Figure 2. Effects of exposure to BPA in adult male BALB/c mice on IL-4 (*A*), IL-10 (*B*), IL-13 (*C*), and IFN-γ (*D*) cytokine production after infection with *L. major.* Values represent mean ± SE ($n = 3$–4).
*$p < 0.05$ and **$p < 0.01$ compared with the nonexposed control group.