containing tetracycline and nystatin (Wako, Osaka, Japan) during the experiment. The Animal Ethics Committee of Shinshu University approved all protocols used in this study.

2.2. Infection with Pneumocystis

Muine *Pneumocystis* was maintained in BALB/c-scid mice (Clea Japan) as described previously [3]. Female wild-type (WT) nude and GKO nude mice were infected with muine *Pneumocystis* by co-housing with infected scid mice. Nine weeks later, they were sacrificed and their lungs were homogenized under aseptic condition. Aliquots of the homogenates were diluted, cytocentrifuged onto glass slides, fixed in methanol, and stained with Fungi-Fluor™ kit (Polysciences, Warrington, PA). The number of cysts in 30 fields was counted by a single investigator under a fluorescent microscope (ECLIPSE E800, Nikon, Tokyo, Japan) as described previously [3]. The lower limit for the detection of cysts was 1.0×10^3/mouse.

2.3. Lung histopathology

Mice were sacrificed 9 weeks after infection by exsanguination following anesthesia with 3.6% chloral hydrate. Lungs were removed and fixed with neutral buffered formalin solution. Paraffin-embedded tissue blocks were then sectioned to 3 μm thickness and stained with hematoxylin and eosin.

2.4. Recovery of bronchoalveolar lavage fuluid (BALF)

Lung airways were lavaged three times with 0.5 ml PBS at 30–37 °C through an intratracheal cannula. BALF were centrifuged at $150 \times g$ for 5 min and the supernatants of the first lavage were stored at −30 °C for determination of cytokines. Cells in BALF were collected by centrifugation and used for intracellular staining.

2.5. In vitro culture of lung cells

Lungs from the infected mice were minced and passed through stainless steel mesh as described previously [15]. After centrifugation collected lung cells were incubated in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% FBS (BioWest, Nuaillé, France) and 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Grand Island, NY), and anti-CD3 mAb at 37 °C for 48 h in an atmosphere of 5% CO_2 and 95% air.

2.6. Determination of cytokines by flow cytometric analysis

Concentrations of cytokines in BALF and culture supernatants were determined using FlowCytomix (Bender Medsystems, Burlingame, CA) and CBA Flex set (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. They were analyzed by FACSCalibur (BD Biosciences).

2.7. Intracellular staining

BALF cells were incubated with PMA and ionomycin in the presence of BD GoldiStop for 5 h. Cells were harvested, and stained with PE-Cy5-labeled anti-CD4 and FITC-labeled anti-CD8 mAbs (BD Biosciences), followed by fixation and permialization with BD Cytofix/Cytoperm. After washing with BD Perm/Wash buffer, cells were stained with PE-labeled anti-IL17 mAb (BD Biosciences). Double-stained cells were analyzed by flow cytometric analysis.

2.8. Statistics

Data were evaluated with the Student's *t*-test for two independent groups. $P < 0.05$ was accepted as indicating significance.

3. Results

3.1. The changes in the body weight and the number of Pneumocystis

WT nude and GKO nude mice were co-housed with *Pneumocystis*-infected SCID mice and weighed every week for evaluating symptoms. Both mice started to lose body weight 7 weeks after infection. WT nude displayed quicker loss of body weight than GKO nude mice. The reduction in body weight of WT nude mice was significantly larger than that in GKO nude mice 8 weeks after infection (Fig. 1A). In contrast, the number of cysts in the lung was significantly smaller in WT nude than in GKO nude mice 9 weeks after infection (Fig. 1B).

3.2. Histopathology in the lung

WT nude mice exhibited pulmonary injury accompanying the infiltration of macrophages, neutrophils and eosinophils into alveoli, hemorrhage and clusters of lymphocytes around the bronchioles (Fig. 2A and B). In GKO nude mice, more hypersensitive pneumonia was observed (Fig. 2C and D). Granulomatous lesions were seen more frequently in GKO nude mice (Fig. 2C). Large numbers of multinucleated giant cells were also observed in them.

3.3. Increased IL-17 production by CD4⁺ T cells in the lung

In order to determine which cytokines are owing to severe inflammatory responses in the lungs of GKO nude mice, levels of cytokines in the BALF were compared between GKO nude and WT nude mice 9 weeks after infection. TNF-α, IL-6 and IL-10 were in BALF of WT nude but scarcely in that of GKO nude mice. In contrast, IL-17 levels in BALF were significantly higher in GKO nude than in WT nude mice. IFN-γ could be detected only in BALF of WT nude mice (Fig. 3A). IL-4 was not detected in both mice (data not shown).

Only a trace of IL-17 and a significant amount of IFN-γ was produced by lung cells in WT nude mice stimulated with anti-CD3 mAb. In contrast, a significant amount of IL-17 was produced by anti-CD3 mAb-stimulated lung cells of GKO nude mice (Fig. 3B and C). To determine which subset of T cells produce IL-17, intracellular staining of BALF cells was carried out. Greater number of BALF CD4⁺ T cells from infected GKO nude mice produced IL-17 than those from WT nude mice (Fig. 4). CD8⁺ T cells from both mice did not show IL-17 production (data not shown).

4. Discussion

In this paper we demonstrated that the number of cysts was greater and histopathology in the lung was severer in GKO nude than in WT nude mice. This suggests that IFN-γ seems to have advantage to *Pneumocystis* infection. Our finding is similar to the results reported previously [16–18]. It has been shown that recombinant IFN-γ enhanced the efficacy of trimethoprim-sulfamethoxazole to resolve PCP in cortisone-treated rats [16]. In addition, exposure to aerosolized IFN-γ significantly lowered *Pneumocystis* burden in CD4⁺ T cell-depleted mice [17]. After primed with IFN-γ in vitro, alveolar macrophages (AM) enhanced their production of nitrogen oxides which are toxic to *Pneumocystis* [18]. Our result was also consistent with reported one that prolonged and exacerbated inflammatory response in lungs was demonstrated in

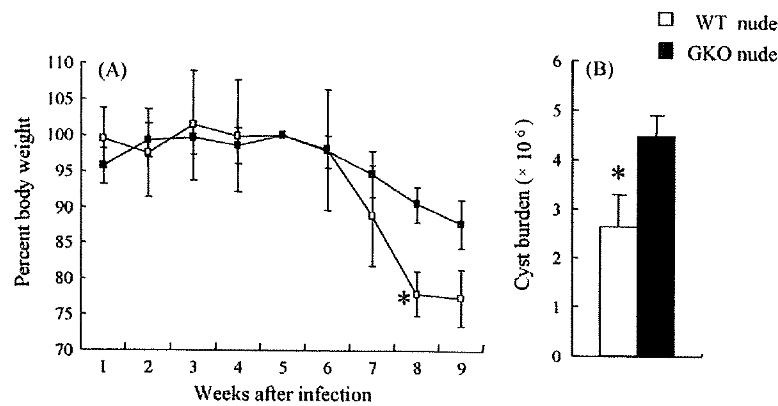

Fig. 1. The changes in the body weight and the number of *Pneumocystis*. GKO nude (■) and WT nude (□) mice were co-housed with *Pneumocystis*-infected scid mice. (A) Mice were weighed every week. Data represented the means ± SD of percent body weights to peak body weight at week 5 after infection ($n = 5$). (B) Lungs of GKO and WT nude mice were homogenized 9 weeks after infection. Aliquots of the homogenates were diluted, cytocentrifuged onto glass slides, fixed in methanol, and stained with Fungi-Fluor kit. Cysts were counted under the fluorescent microscope. Data represent means ± SD ($n = 5$). *Significantly smaller in WT nude mice ($P < 0.05$).

SCID mice inoculated with *Pneumocystis* and reconstituted with splenocytes from GKO mice compared to those reconstituted with splenocytes from WT mice [19]. These results suggest that IFN-γ plays an important role in regulating the inflammatory response to the *Pneumocystis*.

Granulomatous lesions were seen more frequently in GKO nude mice (Fig. 2C), with a large number of multinucleated giant cells. This resembles to the observation reported using a murine model of chlamydial infection, where higher IL-10 production is correlated with lower IFN-γ production, weaker delayed hypersensitivity (DTH), and slower organism clearance followed by granuloma formation at the later stages of infection [20]. Inability of fungicidal activity of alveolar macrophages in GKO nude mice seems to lead the granuloma formation in our experiments. Cases of granulomatous PCP were reported, although not frequently, in HIV and non-HIV patients [21,22]. It is suggested that the pathogenesis of the granulomatous response to *P. jirovecii* may more likely be related to host factors [22], although the precise mechanism is still uncertain. Our experimental model would be a good tool for further investigating the immunological mechanism of granulama formation in PCP.

Our results indicated that greater number of Th17 cells infiltrated and produced higher levels of IL-17 in the lungs of *Pneumocystis*-infected GKO nude than in WT nude mice. Th1 immune response took the place of Th17 response in WT nude mice. IL-17 was known as a proinflammatory cytokine that mediates multiple chronic inflammatory responses including angiogenesis, recruitment of inflammatory cells, and induction of proinflammatory mediators by endothelial and epithelial tissues [23]. IFN-γ was considered to suppress Th17 differentiation in our experimental model, as was also reported previously [24]. Upregulated production of IL-17 must be the factors of the severer PCP in GKO nude

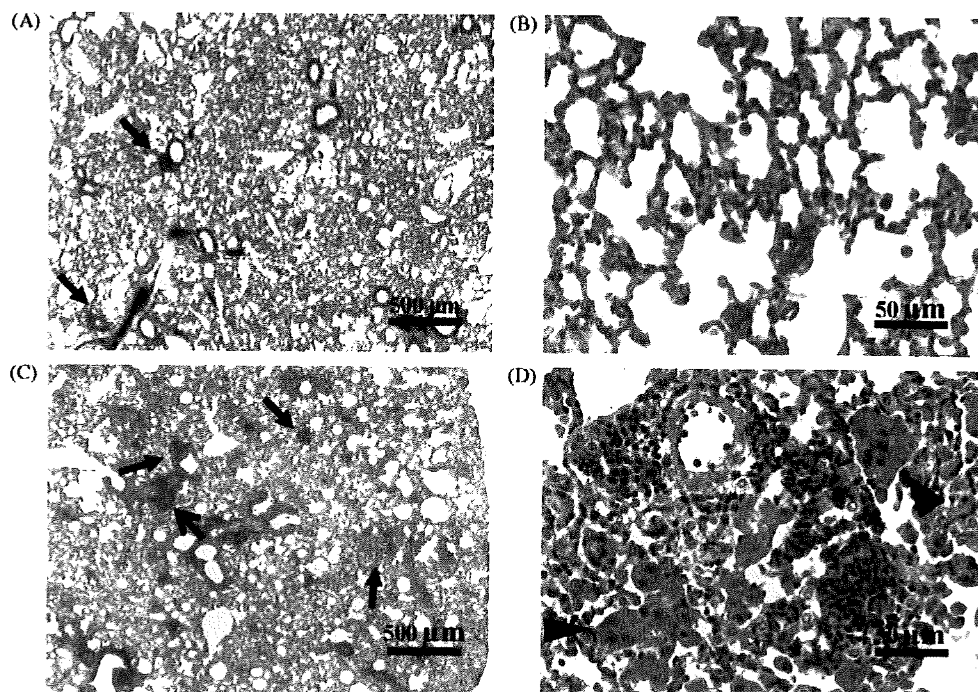

Fig. 2. Sections of lungs from WT nude (A and B) and GKO nude (C and D) mice were stained with hematoxylin and eosin 9 weeks after infection. Arrows indicate granulomatous lesion (A and C). Arrowheads indicate multinucleated giant cells (D).

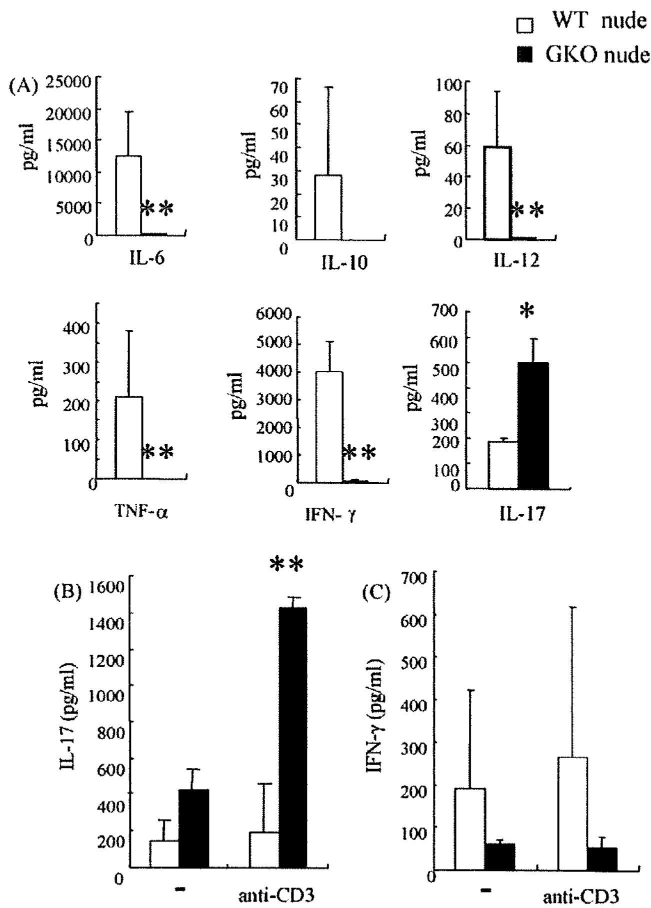

Fig. 3. Levels of cytokines in BALF of GKO nude and WT nude mice 9 weeks after infection. (A) Lung airways were lavaged with 0.5 ml PBS through an intratracheal cannula and concentrations of cytokines in the first lavage were determined. Data represent means ± SD ($n = 5$). IL-17 (B) and IFN-γ (C) production by lung T cells from GKO and WT nude mice. Lung cells from infected GKO and WT nude mice were incubated with anti-CD3 mAb for 48 h. Concentrations of IL-17 and IFN-γ in the supernatant were determined. Data represent means ± SD ($n = 5$). *Significantly different from WT nude mice ($P < 0.05$). **Significantly different from WT nude mice ($P < 0.01$).

mice, although the loss of body weight was greater in WT nude than in GKO nude mice. This may be because TNF-α production was also reduced in GKO nude mice. This should be addressed in the future study.

The roles of IL-17 in fungal infection remain controversial. It has recently been reported that IL-23/IL-17 developmental pathway negatively regulates Th1 responses to *Aspergillus fumigatus* and *Candida albicans* and permits more extensive growth of fungi in vivo. Moreover, IL-17 inhibits antifungal activity in vitro [25]. In contrast, lower levels of IL-17 in the lung caused by IL-23 deficiency or neutralization with anti-IL-17 mAb resulted in transient decrease (3 weeks after infection) in clearance of *Pneumocystis* organisms [26]. $CD4^+$ T cells were not depleted and *Pneumocystis* were cleared afterwards in their experiments, suggesting that IL-17 might have fungicidal activity but is not necessary for clearance of *Pneumocystis* organisms in immunocompetent hosts. In our study, the number of Th17 cells and production of IL-17 increased in the lungs of GKO nude mice, although *Pneumocystis* burden in the lung was not decreased compared with WT nude ones. This suggested that Th17 cells seem not to be effective to protect *Pneumocystis* infection in immunocompromised hosts.

IRIS has been well described in AIDS patients receiving antiretroviral therapy. This paradoxical worsening of clinical symptoms after immunological recovery has been associated with an excessive inflammatory response to either intercurrent or previously unrecognised (subclinical) opportunistic infections [27]. There are reports of similar paradoxical symptoms occurring in patients without HIV infection following chemotherapy for *Mycobacterium* species infections [9] or cessation of a TNF-α blocker [28]. Because few longitudinal sample sets are available to characterize the immune response, the immunopathology of IRIS is poorly understood.

$CD25^+CD4^+$ Treg cells has been reported to control pulmonary inflammation and lung injury in IRIS with *Pneumocystis* infection [6,29]. Transfer of $CD25^-CD4^+$ T cells without $CD25^+CD4^+$ Treg cells to *Pneumocystis*-infected SCID resulted in hyperinflammatory response that lead to lung injury and death with strong IFN-γ in the lung [29]. Increased levels of IFN-γ and chemokines induced by IFN-γ such as IP-10 in IRIS patients were also reported [30,31]. These data suggested that there might be a deficit in the ability to control or turn off antigen-specific immune responses following antigen clearance. Absence of Treg cells results in severe deregulation of the immune system, leading to lymphoproliferation and autoimmune disease [32]. Therefore, it is considered that Treg cells might be deficient in numbers or in function in IRIS patients. Instead Treg cells increased at the peak of IRIS symptoms [33]. In addition, a significant expansion of $CD127^{lo}Foxp3^+CD25^+$ Treg cells was observed in IRIS patients compared with healthy controls and also compared with late-stage HIV-infected patients who commenced combination antiretroviral therapy without developing an IRIS [31]. It is important to clarify the role of Treg cells in IRIS patients.

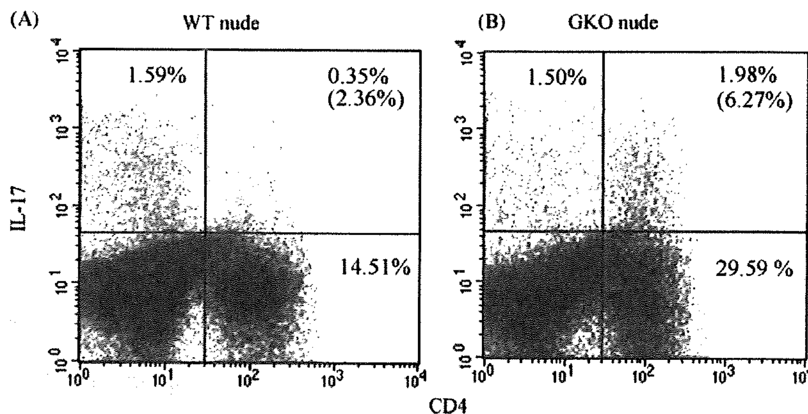

Fig. 4. Intracellular staining of $CD4^+$ T cells from BALF of GKO and WT nude mice. Percentages in the parentheses indicate the percentage of IL-17 producing $CD4^+$ cells in $CD4^+$ T cells.

Our results clearly demonstrated using a nude mouse model that deficiency in IFN-γ induces the differentiation of Th17 and that IL-17 is responsible for inflammatory response in PCP. Introducing modified gene into nude animals would be a good tool for evaluating inflammatory reactions against *Pneumocystis* and other opportunistic infections.

References

[1] Harmsen AG, Stankiewicz M. Requirement for CD4+ cells in resistance to *Pneumocystis carinii* pneumonia in mice. J Exp Med 1990;17:2937–45.

[2] Phair J, Muñoz A, Detels R, Kaslow R, Rinaldo C, Saah A. The risk of *Pneumocystis carinii* pneumonia among men infected with human immunodeficiency virus type 1, Multicenter AIDS Cohort Study Group. N Engl J Med 1990;322:161–5.

[3] Ding K, Shibui A, Wang Y, Takamoto M, Matsuguchi T, Sugane K. Impaired recognition by Toll-like receptor 4 is responsible for exacerbated murine *Pneumocystis* pneumonia. Microbes Infect 2005;7:195–203.

[4] Walzer PD, Powell Jr RD, Yoneda K. Experimental *Pneumocystis carinii* pneumonia in different strains of cortisonized mice. Infect Immun 1979;24:939–47.

[5] Roths JB, Sidman CL. Both immunity and hyperresponsiveness to *Pneumocystis carinii* result from transfer of CD4+ but not CD8+ T cells into severe combined immunodeficiency mice. J Clin Invest 1992;90:673–8.

[6] Hori S, Carvalho LT, Demengeot J. CD25+CD4+ regulatory T cells suppress CD4+ T cell mediated pulmonary hyperinflammation driven by *Pneumocystis carinii* in immunodeficient mice. Eur J Immunol 2002;32:1282–91.

[7] Barry SM, Lipman MC, Deery AR, Johnson MA, Janossy G. Immune reconstitution pneumonitis following *Pneumocystis carinii* pneumonia in HIV-infected subjects. HIV Med 2002;3:207–11.

[8] Beck JM, Rosen MJ, Peavy HH. Pulmonary complications of HIV infection: report of the Fourth NHLBI Workshop. Am J Respir Crit Care Med 2001;164:2120–6.

[9] Cheng VC, Ho PL, Lee RA, Chan KS, Chan KK, Woo PC, et al. Clinical spectrum of paradoxical deterioration during antituberculosis therapy in non-HIV-infected patients. Eur J Clin Microbiol Infect Dis 2002;21:803–9.

[10] Delclaux C, Zahar JR, Amraoui G, Leleu G, Lebargy F, Brochard L, et al. Corticosteroids as adjunctive therapy for severe *Pneumocystis carinii* pneumonia in non-human immunodeficiency virus-infected patients: retrospective study of 31 patients. Clin Infect Dis 1999;29:670–2.

[11] Kennedy JD, Pierce CW, Lake JP. Extrathymic T cell maturation. Phenotypic analysis of T cell subsets in nude mice as a function of age. J Immunol 1992;148:1620–9.

[12] Takamoto M, Sugane K. Mechanisms of eosinophilia in *Toxocara canis* infected mice: *in vitro* production of Interleukin 5 by lung cells of both normal and congenitally athymic nude mice. Parasite Immunol 1993;15:493–500.

[13] Tagawa Y, Sekikawa K, Iwakura Y. Suppression of concanavalin A-induced hepatitis in IFN-$\gamma^{-/-}$ mice, but not in TNF-$\alpha^{-/-}$ mice: role for IFN-γ in activating apoptosis of hepatocytes. J Immunol 1997;159:1418–28.

[14] Tagawa Y, Matthys P, Heremans H, Dillen C, Zaman Z, Iwakura Y, et al. Bimodal role of endogenous interleukin-6 in concanavalin A-induced hepatitis in mice. J Leukoc Biol 2000;67:90–6.

[15] Takamoto M, Kusama Y, Takatsu K, Nariuchi H, Sugane K. Occurrence of interleukin-5 production by CD4−CD8− (double negative) T cells in lungs of both normal and congenitally athymic nude mice infected with *Toxocara canis*. Immunology 1995;85:285–91.

[16] Shear HL, Valladares G, Narachi MA. Enhanced treatment of *Pneumocystis carinii* pneumonia in rats with interferon-γ and reduced doses of trimethoprim/sulfamethoxazole. J Acquir Immune Defic Syndr 1990;3:943–8.

[17] Beck JM, Liggitt HD, Brunette EN, Fuchs HJ, Shellito JE, Debs RJ. Reduction in intensity of *Pneumocystis carinii* pneumonia in mice by aerosol administration of gamma interferon. Infect Immun 1991;59:3859–62.

[18] Downing JF, Kachel DL, Pasula R, Martinll WJ. Gamma interferon stimulates rat alveolar macrophages to kill *Pneumocystis carinii* by L-arginine and tumor necrosis factor-dependent mechanisms. Infect Immun 1999;67:1347–52.

[19] Garvy BA, Ezekowitz RA, Harmsen AG. Role of gamma interferon in the host immune and inflammatory responses to *Pneumocystis carinii* infection. Infect Immun 1997;65:373–9.

[20] Yang X, Gartner J, Zhu L, Wang S, Brunham RC. IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following *Chlamydia trachomatis* lung infection. J Immunol 1999;162:1010–7.

[21] Bondoc AY, White DA. Granulomatous *Pneumocystis carinii* pneumonia in patients with malignancy. Thorax 2002;57:435–7.

[22] Totet A, Duwat H, Daste G, Berry A, Escamilla R, Nevez G. *Pneumocystis jirovecii* genotypes and granulomatous pneumocystosis. Méd mal infect 2006:36229–31.

[23] Pesanti EL. *Pneumocystis carinii* oxygen uptake, antioxidant enzymes, and susceptibility to oxygen-mediated damage. Infect Immun 1984;44:7–11.

[24] Mandy JM, Daniel JC. Th17 cell differentiation: the long and winding road. Immunity 2008;28:445–53.

[25] Zelante T, De Luca A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, et al. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol 2007;10:2695–706.

[26] Rudner XL, Happel KI, Young EA, Shellito JE. Interleukin-23 (IL-23)-IL-17 cytokine axis in murine *Pneumocystis carinii* infection. Infect Immun 2007;75:3055–61.

[27] Hirsch HH, Kaufmann G, Sendi P, Battegay M. Immune reconstitution in HIV-infected patients. Clin Infect Dis 2004;38:1159–66.

[28] Cadena J, Thompson III GR, Ho TT, Medina E, Hughes DW, Patterson TF. Immune reconstitution inflammatory syndrome after cessation of the tumor necrosis factor α blocker adalimumab in cryptococcal pneumonia. Diagn Microbiol Infect Dis 2009;64:327–30.

[29] McKinley L, Logar AJ, McAllister F, Zheng M, Steele C, Kolls JK. Regulatory T cells dampen pulmonary inflammation and lung injury in an animal model of pneumocystis pneumonia. J Immunol 2006;177:6215–26.

[30] Bourgarit A, Carcelain G, Martinez V, Lascoux C, Delcey V, Gicquel B, et al. Explosion of tuberculin-specific Th1-responses induces immune restoration syndrome in tuberculosis and HIV co-infected patients. AIDS 2006;20:1–7.

[31] Seddiki N, Sasson SC, Santner-Nanan B, Munier M, van Bockel D, Ip S, et al. Proliferation of weakly suppressive regulatory CD4+ T cells is associated with over-active CD4+ T-cell responses in HIV-positive patients with mycobacterial immune restoration disease. Eur J Immunol 2009;39:391–403.

[32] Ochs HD, Ziegler SF, Torgerson TR. FOXP3 acts as a rheostat of the immune response. Immunol Rev 2005;203:156–64.

[33] Tan DB, Yong YK, Tan HY, Kamarulzaman A, Tan LH, Lim A, et al. Immunological profiles of immune restoration disease presenting as mycobacterial lymphadenitis and cryptococcal meningitis. HIV Med 2008;9:307–16.

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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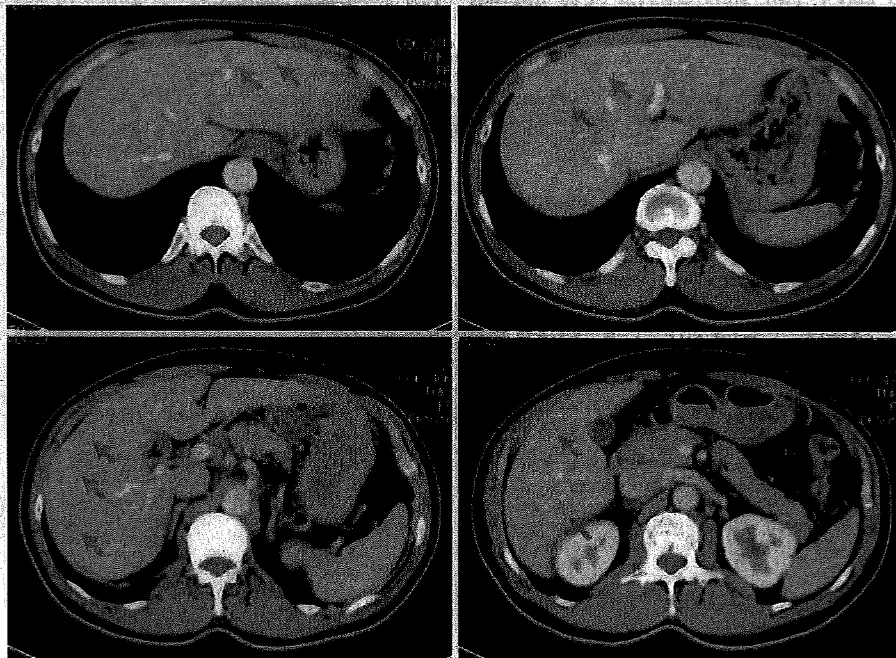

Picture 1. Contrast-enhanced CT scan image obtained at portal venous phase showing multiple small, ill-defined, and low-attenuation lesions in the liver of the patient (arrows).

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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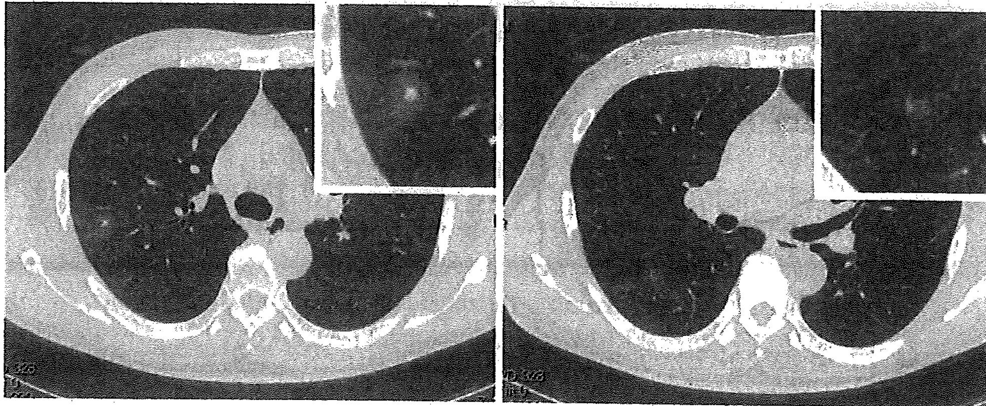

Picture 2. Chest CT scan image showing nodule with halo and ill-defined margin (left), and groundglass opacity with an ill-defined margin (right). The lesion is shown magnified in the inset.

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.

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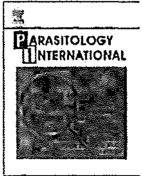

Case report

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

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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.

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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.

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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%

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 (×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 (×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.

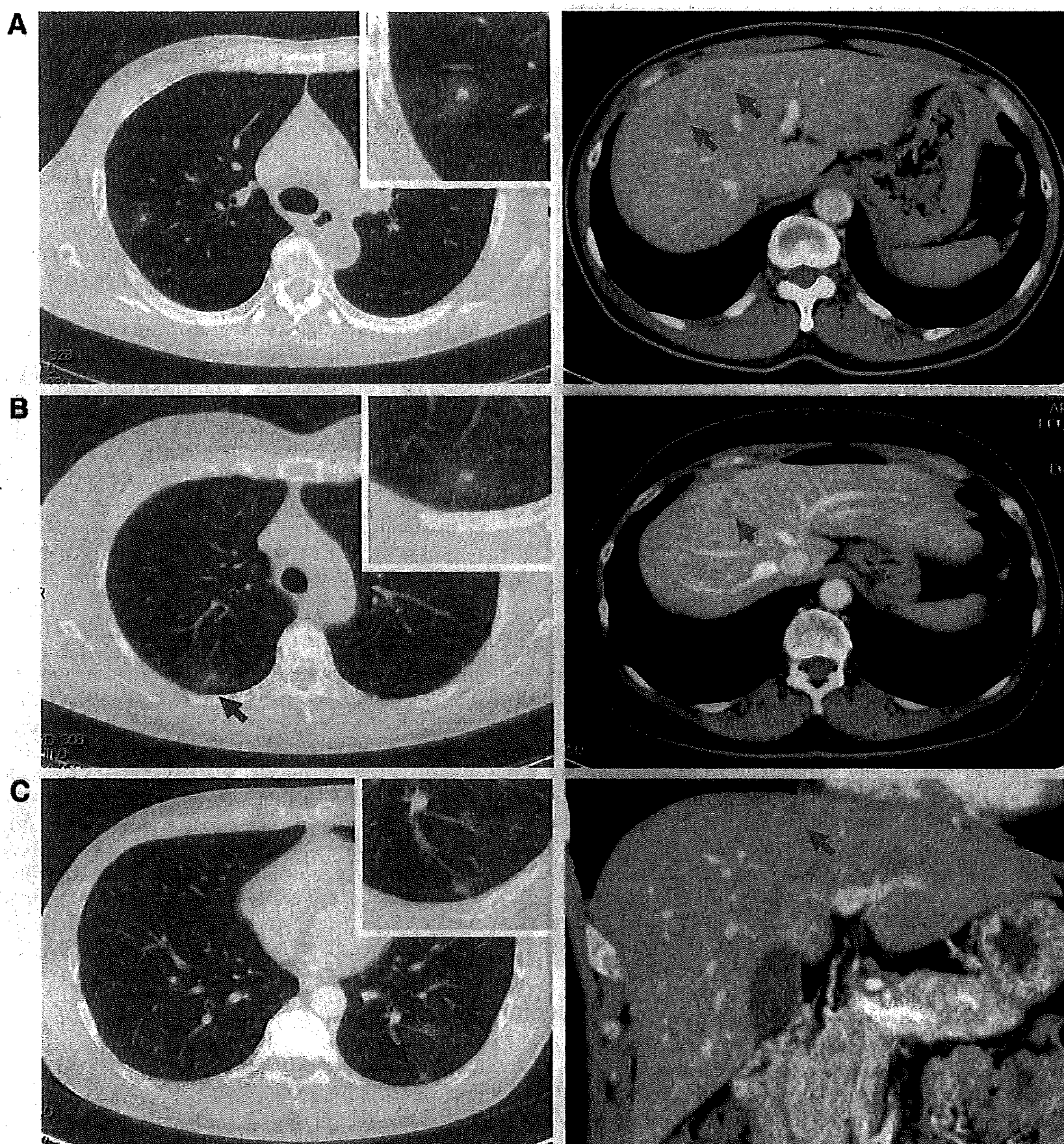

Fig. 1. CT images of pulmonary and hepatic lesions. A, B, and C show representative CT images from Patients 1, 2, and 3, respectively. Pulmonary lesions (arrows, magnified in inset) were shown as nodules with halos and a poorly defined margin or ground-glass opacity with a poorly defined margin. Hepatic 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*

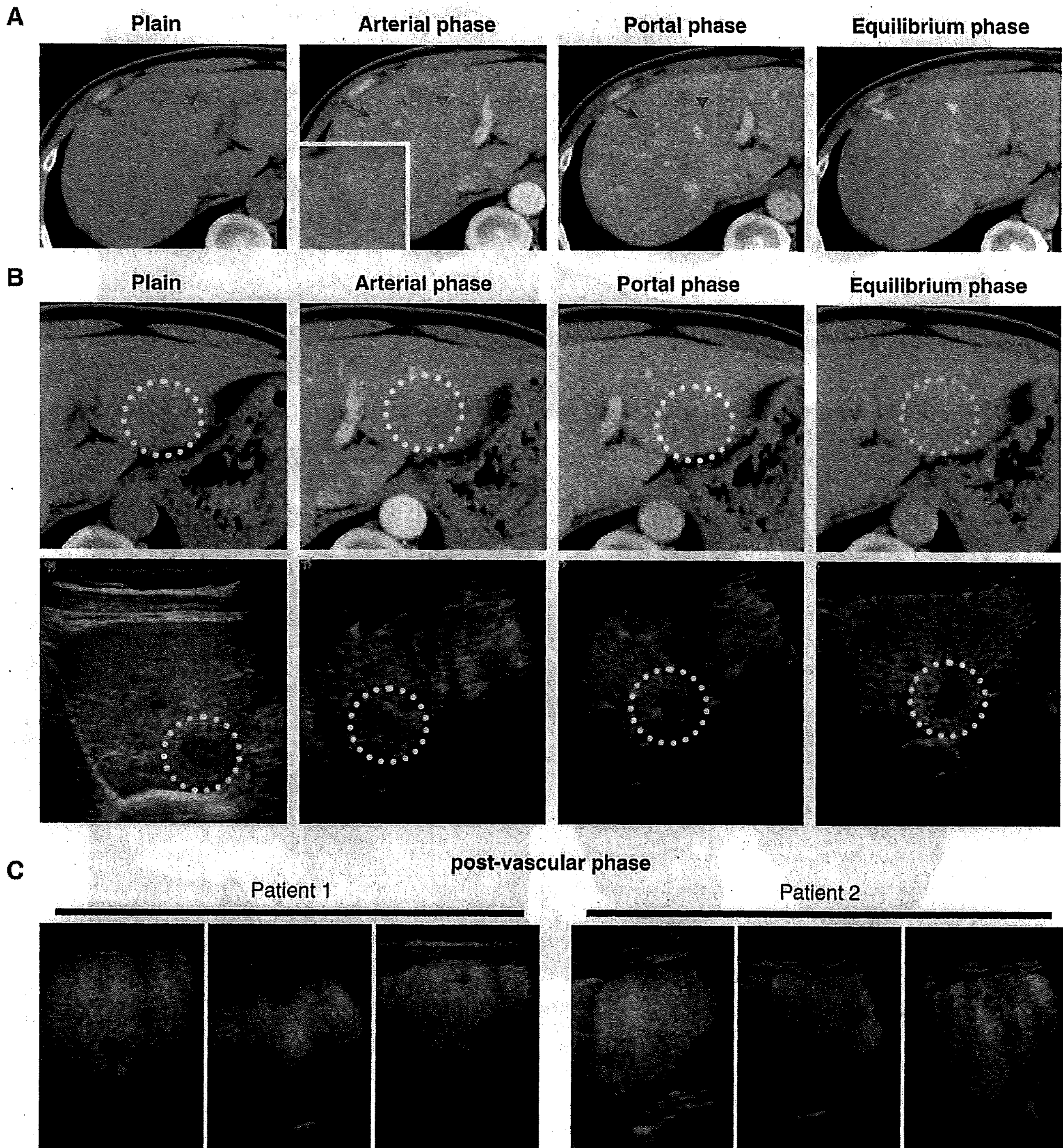

Fig. 2. CT and US images of hepatic lesions. A. Two nodules (arrow and arrowhead) in the liver of Patient 1 are shown. That shown by the arrowhead (magnified in inset) had weak peripheral rim enhancement in the arterial phase, while both were undetectable in the equilibrium phase. B. A lesion (circle 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

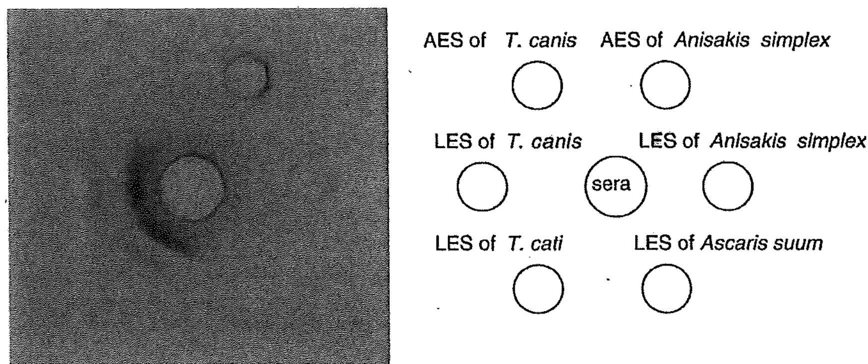

Fig. 3. Results of serological tests. Data from Patient 1 shown with schematic positions of antigens and sera are presented as representative findings. The antigens used were adult worm extract (AES) of *T. canis*, AES of *Anisakis simplex*, larval excretory–secretory (LES) of *Anisakis simplex*, LES 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.

References

[1] Beaver PC, Jung RC, Cupp EW. Clinical parasitology 9 Philadelphia. Lea & Febiger: PA; 1984. p. 320–9.
[2] Glickman LT, Schantz PM. Epidemiology and pathogenesis of zoonotic toxocariasis. Epidemiol Rev 1981;3:230–50.
[3] Kondo K. Toxocara infection and toxocariasis. In: Kamegai S, Hayashi S, editors. Progress of medical parasitology in Japan Tokyo: Meguro Parasitological Museum; 2003. p. 475–84.
[4] Despommier D. Toxocariasis: clinical aspects, epidemiology, medical ecology, and molecular aspects. Clin Microbiol Rev 2003;16:265–72.
[5] Magnaval JF, Glickman LT, Dorchies P, Morassin B. Highlights of human toxocariasis. Korean J Parasitol 2001;39:1–11.
[6] Akao N, Ohta N. Toxocariasis in Japan. Parasitol Int 2007;56:87–93.
[7] Sakai S, Shida Y, Takahashi N, Yabuuchi H, Soeda H, Okafuji T, et al. Pulmonary lesions associated with visceral larva migrans due to *Ascaris suum* or *Toxocara canis*: imaging of six cases. AJR Am J Roentgenol 2006;186:1697–702.
[8] Chang S, Lim JH, Choi D, Park CK, Kwon NH, Cho SY, et al. Hepatic visceral larva migrans of *Toxocara canis*: CT and sonographic findings. AJR Am J Roentgenol 2006;187:W622–9.
[9] Lim JH. Toxocariasis of the liver: visceral larva migrans. Abdom Imaging 2008;33:151–6.
[10] Kindberg GM, Tolleshaug H, Roos N, Skotland T. Hepatic clearance of Sonazoid perfluorobutane microbubbles by Kupffer cells does not reduce the ability of liver to phagocytose or degrade albumin microspheres. Cell Tissue Res 2003;312:49–54.
[11] Akao N, Chu AE, Tsukidate S, Fujita K. A rapid and sensitive screening kit for the detection of anti-Toxocara larval ES antigens. Parasitol Int 1997;46:189–95.
[12] Beaver PC, Synder CH, Carrera GM. Chronic eosinophilia due to visceral larva migrans. Pediatrics 1952;9:7–19.
[13] Sakai K, Okajima Y, Ohuchi K. A case of visceral larva migrans due to the ingestion of raw hen liver. Naika 1983;51:963–7 [in Japanese with English abstract].
[14] Ito K, Sakai K, Okajima T, Ouchi K, Funakoshi A, Nishimura J, et al. Three cases of visceral larva migrans due to ingestion of raw chicken or cow liver. J Jpn Soc Int Med 1986;75:759–66 [in Japanese].
[15] Nakatsuji Y, Shigemoto S, Kojiro N, Nanahoshi M, Masaki S. Brother cases of serologically diagnosed visceral larva migrans. J Jpn Soc Int Med 1989;78:35–40 [in Japanese].
[16] Nagakura K, Tachibana H, Kaneda Y, Kato Y. Toxocariasis possibly caused by ingesting raw chicken. J Infect Dis 1989;160:735–6.
[17] Morimatsu Y, Akao N, Akiyoshi H, Kawazu T, Okabe Y, Aizawa H. A familial case of visceral larva migrans after ingestion of raw chicken livers: appearance of specific antibody in bronchoalveolar lavage fluid of the patients. Am J Trop Med Hyg 2006;75:303–6.
[18] Mitamura M, Fukuoka M, Haruta Y, Koarada S, Tada Y, Nagasawa K. A case of visceral larva migrans due to *Toxocara canis* showing varied manifestations. Kansenshogaku Zasshi 2007;81:305–8 [in Japanese with English abstract].
[19] Hoffmeister B, Glaeser S, Flick H, Pornschlegel S, Suttorp N, Bergmann F. Cerebral toxocariasis after consumption of raw duck liver. Am J Trop Med Hyg 2007;76:600–2.
[20] Salem G, Schantz P. Toxocaral visceral larva migrans after ingestion of raw lamb liver. Clin Infect Dis 1992;15:743–4.
[21] Sturchler D, Weiss N, Gassner M. Transmission of toxocariasis. J Infect Dis 1990;162:571.
[22] Taira K, Saeed I, Permin A, Kapel CM. Zoonotic risk of *Toxocara canis* infection through consumption of pig or poultry viscera. Vet Parasitol 2004;7:115–24.
[23] Taira K, Permin A, Kapel CM. Establishment and migration pattern of *Toxocara canis* larvae in chickens. Parasitol Res 2003;90:521–3.
[24] Lee KT, Min HK, Chung PR, Chang JK. Studies on the inducing possibility of human visceral larva migrans associated with eating habit of raw liver of domestic animals. Korean J Parasitol 1976;14:51–60.
[25] Fan CK, Lan HS, Hung CC, Chung WC, Liao CW, Du WY, et al. Seroepidemiology of *Toxocara canis* infection among mountain aboriginal adults in Taiwan. Am J Trop Med Hyg 2004;71:216–21.
[26] Baixench MT, Magnaval JF, Dorchies P. Epidémiologie de la toxocarose chez les étudiants de l'Ecole Nationale Vétérinaire de Toulouse. Rev Med Vet 1992;143:749–52.
[27] Takakura Y. An epidemiological study of food-borne toxocariasis: fowl and cattle as paratenic hosts of *Toxocara canis*. J Juzen Med 1993;102:828–35 [in Japanese with English abstract].
[28] Olsen A, Permin A, Roepstorff A. Chickens and pigs as transport hosts for *Ascaris*, *Trichuris* and *Oesophagostomum* eggs. Parasitology 2001;123:325–30.
[29] Permin A, Henningsen E, Murrell KD, Roepstorff A, Nansen P. Pigs become infected after ingestion of livers and lungs from chickens infected with *Ascaris* of pig origin. Int J Parasitol 2000;30:867–8.
[30] Kwon NH, Oh MJ, Lee SP, Lee BJ, Choi DC. The prevalence and diagnostic value of toxocariasis in unknown eosinophilia. Ann Hematol 2006;85:233–8.
[31] Sturchler D, Schubarth P, Gualzata M, Gottstein B, Oettli A. Thiabendazole vs. albendazole in treatment of toxocariasis: a clinical trial. Ann Trop Med Parasitol 1989;83:473–8.
[32] Hotez PJ. *Toxocara canis*. In: Burg FD, Wald ED, Ingelfinger JR, Polin PA, editors. Gellis and Kagan's current pediatric therapy. 15th ed. Philadelphia, PA: W. B. Saunders Pubs.; 1995. p. 683–4.
[33] Drugs for parasitic infections. Med Lett Drugs Ther 2004;1189:1–12.
[34] Hossack J, Ricketts P, Te HS, Hart J. A case of adult hepatic toxocariasis. Nat Clin Pract Gastroenterol Hepatol 2008;5:344–8.
[35] Matsuki Y, Fujii T, Nakamura-Uchiyama F, Hiromatsu K, Nawa Y, Hayashi T, et al. Toxocariasis presenting with multiple effusions in the pericardial space, thoracic cavity, and Morrison's pouch. Intern Med 2007;46:913–4.
[36] Altcheh J, Nallar M, Conca M, Biancardi M, Freilij H. Toxocariasis: clinical and laboratory features in 54 patients. An Pediatr (Barc) 2003;58:425–31 [in Spanish with English abstract].
[37] Inoue K, Inoue Y, Arai T, Nawa Y, Kashiwa Y, Yamamoto S, et al. Chronic eosinophilic pneumonia due to visceral larva migrans. Intern Med 2002;41:478–82.
[38] Sauvet F, Graffin B, Leyral G, Chemsi M, Paris JF, Carli P. Pulmonary eosinophilia in France: possible toxocariasis. Presse Med 2005;34:1713–4 [in French with English abstract].
[39] Huh S, Lee S. Toxocariasis. http://www.emedicine.com/med/topic2293.htm.

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

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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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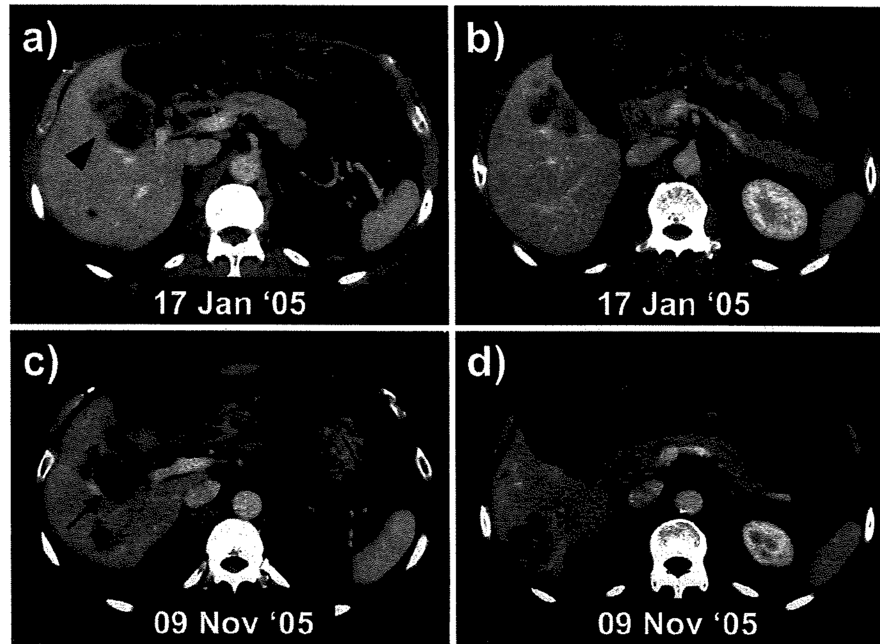

Figure 1. The contrast-enhanced CT image presented huge cystic and multilocular lesions. a, b) In the anterior segment of the right lobe, a huge and low-attenuated mass (arrowhead) and some tiny hypodense lesions were detected. c) The corresponding lesion in the anterior segment migrated into the center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

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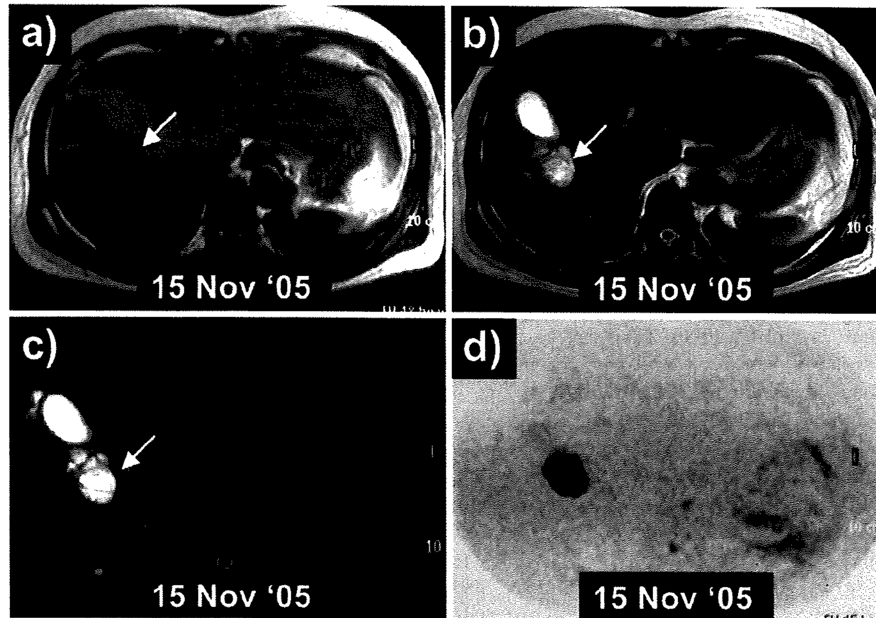

Figure 2. The corresponding MR image showed necrotic or abscess-forming lesions (arrows); a) T1-weighted images, b) T2-weighted images, c) fat-suppressed T2-weighted image, d) inverted diffusion-weighted images.

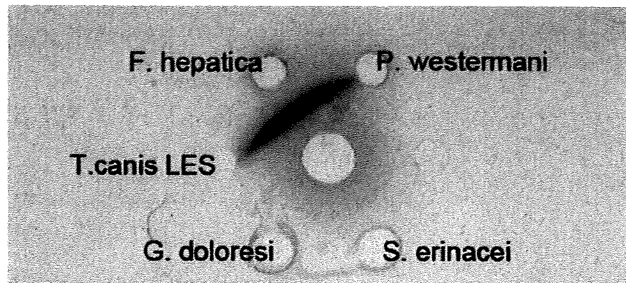

Figure 3. The ouchterlony double-diffusion test showed a strong precipitin band against crude antigen on *Fasciola hepatica*. The positions of antigens; F. hepatica, *Fasciola hepatica*; P. westermani, *Paragonimus westermani*; T. canis LES, Larval excretory and secretory antigen of *Toxocara canis*;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

References

1. Mas-Coma S. Epidemiology of fascioliasis in human endemic areas. J Helminthol **79**: 207-216, 2005.
2. Aksoy DY, Kerimoglu U, Oto A, et al. *Fasciola hepatica* infection: Clinical and computerized tomographic findings of ten patients. Turk J Gastroenterol **17**: 40-45, 2006.
3. Pulpeiro JR, Armesto V, Varela J, Corredoira J. Fascioliasis: findings in 15 patients. Br J Radiol **64**: 798-801, 1991.
4. Yamaura H, Araki K, Kikuchi K, Itoda I, Totsuka K, Kobayakawa T. Evaluation of dot-ELISA for serological diagnosis of amebiasis. J Infect Chemother **9**: 25-29, 2003.
5. Keiser J, Engels D, Buscher G, Utzinger J. Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Investig Drugs **14**: 1513-1526, 2005.
6. Arjona R, Riancho JA, Aguado JM, Salesa R, González-Macías J. Fascioliasis in developed countries: a review of classic and aberrant forms of the disease. Medicine **74**: 13-23, 1995.
7. Kabaalioglu A, Cubuk M, Senol U, et al. Fascioliasis: US, CT, and MRI findings with new observations. Abdom Imaging **25**: 400-404, 2000.
8. Van Beers B, Pringot J, Geubel A, et al. Hepatobiliary fascioliasis: noninvasive imaging findings. Radiology **174**: 809-810, 1990.
9. Gonzalez Llorente J, Herrero Domingo A, Carrero Gonzalez P. Subcapsular abscess: an unusual CT finding in hepatic fascioliasis. Am J Roentgenol **178**: 514-515, 2002.
10. Kim KA, Lim HK, Kim SH, Lee WJ, Lim JH. Necrotic granuloma of the liver by human fascioliasis: imaging findings. Abdom Imaging **24**: 462-464, 1999.
11. Mortele KJ, Ros PR. Cystic focal liver lesions in the adult: differential CT and MR imaging features. Radiographics **21**: 895-910, 2001.
12. Han JK, Han D, Choi BI, Han MC. MR findings in human fascioliasis. Trop Med Int Health **1**: 367-372, 1996.
13. Cevikol C, Karaali K, Senol U, et al. Human fascioliasis: MR imaging findings of hepatic lesions. Eur Radiol **13**: 141-148, 2003.
14. Mortele KJ, Ros PR. Cystic focal liver lesions in the adult: differential CT and MR imaging features. Radiographics **21**: 895-910, 2001.

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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.

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.

LITERATURE CITED

Abdel-Hameed, A. A. 1984. Effect of thiabendazole on the migration of *Toxocara canis* larvae in the mouse. Journal of Parasitology **70:** 226–231.

Fok, E., and T. Kassai. 1998. *Toxocara canis* infection in the paratenic host: A study on the chemosusceptibility of the somatic larvae in mice. Veterinary Parasitology **74:** 243–259.

Havasiova-Reiterova, K., O. Tomasovicova, and P. Dubinsky. 1995. Effect of various doses of infective *Toxocara canis* and *Toxocara cati* eggs on the humoral response and distribution of larvae in mice. Parasitology Research **81:** 13–17.

Horiuchi, A., T. Satou, N. Akao, K. Koike, K. Fujita, and T. Nikaido. 2005. The effect of free and polyethylene glycol-liposome-entrapped albendazole on larval mobility and number in *Toxocara canis* infected mice. Veterinary Parasitology **129:** 83–87.

Hrckova, G., and S. Velebny. 2001. Treatment of *Toxocara canis* infections in mice with liposome-incorporated benzimidazole carbamates and immunomodulator glucan. Journal of Helminthology **75:** 141–146.

Oshima, T. 1961. Influence of pregnancy and lactation on migration of the larvae of *Toxocara canis* in mice. Journal of Parasitology **47:** 657–660.

Satou, T., A. Horiuchi, N. Akao, K. Koike, K. Fujita, and T. Nikaido. 2005. *Toxocara canis*: Search for a potential drug amongst beta-carboline alkaloids—In vitro and mouse studies. Experimental Parasitology **110:** 134–139.

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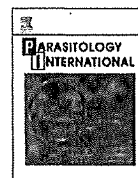

Prolactin evokes lactational transmission of larvae in mice infected with *Toxocara canis*

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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.

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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 ($P<0.05$).

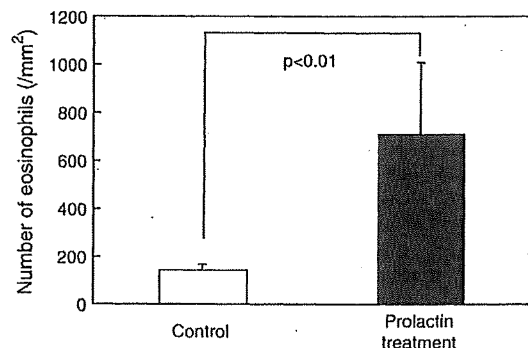

Fig. 1. Eosinophil counts around the capsules of mammary glands in mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 713.6±293.6 cells/mm^2 in the prolactin-treated group, and 144±21.3 cells/mm^2 in the saline-treated 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.

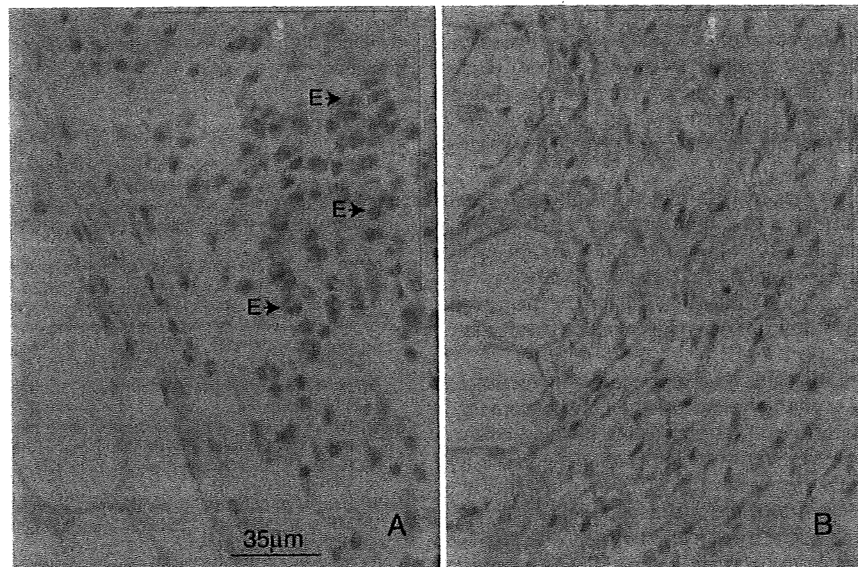

Fig. 2. Histopathological findings of mammary glands around the connective tissue in mice. Serial sections of mammary glands of female mice were stained with haematoxylin and eosin. Markedly higher eosinophilic (E) infiltrations around the connective tissue of the mammary gland were observed in the prolactin-treated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

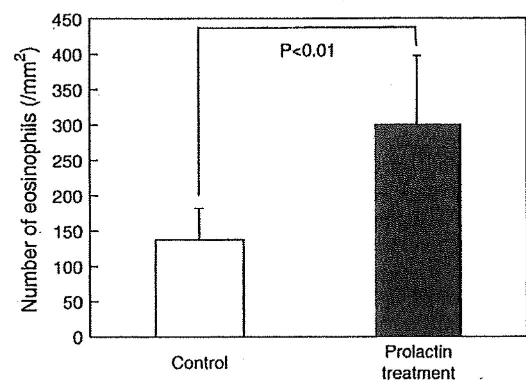

Fig. 3. Eosinophil counts around the capsules of mammary glands in chronically infected mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 300.8±95.6 cells/mm^2 in the prolactin-treated group, and 137.6±44.1 cells/mm^2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