



Parasite-specific antibody profile in the aqueous humor of rabbits with ocular toxocariasis

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ABSTRACT

Human ocular toxocariasis is diagnosed using ophthalmologic and immunologic examinations. Many researchers have suggested that intraocular parasite-specific antibody levels are indicative of ocular toxocariasis, but little is known about the time course of the changes in these levels. We therefore investigated the anti-*Toxocara canis* antibody profile in the aqueous humor in an animal model of ocular toxocariasis. We intravitreally injected *T. canis* larvae into the right eye of 4 rabbits; 2 rabbits were orally administered *T. canis* eggs. We collected serum, aqueous humor, and tear samples weekly and determined the serum and aqueous humor levels of anti-*T. canis* immunoglobulin (Ig)G, IgA, IgM, and IgE antibodies and the tear IgG antibody level by enzyme-linked immunosorbent assay (ELISA). The severity of vitreous opacity and the aqueous humor IgG levels (measured using optical density [OD]) changed concordantly in the larvae-injected eyes; the OD exceeded 0.1 from 2–4 weeks after infection and remained elevated during active intraocular inflammation. However, the aqueous humor IgG levels were also elevated in 6 out of 8 eyes without intraocular larvae in both groups, and were low in 1 eye with live intravitreal larvae. In contrast, the serum IgG and IgM levels and the tear IgG levels increased in all rabbits, regardless of the presence of intraocular inflammation. Vitreous opacity occurred in all intravitreally infected eyes, but significant histopathological evidence of retinal damage was not detected. Thus, besides the presence of intraocular larvae, some other factors in the host may be required for the development of retinal lesions.

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1. Introduction

Human toxocariasis is a helminthozoonosis that occurs worldwide and is caused by infestation with the larvae of the nematode worms *Toxocara canis* or *Toxocara cati* [1]. Toxocariasis is clinically classified into 4 types: visceral, ocular, neurological, and covert [2]. Ocular toxocariasis is an uncommon cause of uveitis [3,4], and pars plana vitrectomy has been used effectively to remove vitreous opacities and eliminate vitreoretinal traction [5–9]. However, ocular toxocariasis remains an important differential diagnosis of retrolental fibroplasia, congenital retinal fold, pars planitis, toxoplasmosis, histoplasmosis, Coats disease, retinoblastoma, etc. [2,10].

Wilder first observed intraocular nematodiasis in 1950 during pathological examination of enucleated eyes; in most instances, enucleation was performed following a clinical diagnosis of retino-

blastoma [11]. In 1956, Nichols identified these nematodes as the second-stage larvae of *T. canis* [12]. Since then, many cases of ocular toxocariasis that were diagnosed because of the presence of larvae in enucleated eyes have been reported [13–15]. In 1971, Wilkinson and Welch classified ocular toxocariasis into (1) diffuse nematode endophthalmitis, (2) posterior pole granuloma, and (3) peripheral inflammatory mass [10]. However, this classification is not applicable to all cases because of variations in the clinical presentation of ocular toxocariasis.

Patients with toxocariasis do not excrete parasitic materials such as eggs or larvae; toxocariasis is confirmed by the histopathological detection of larvae in the affected tissues. Larval fragments are occasionally recovered from vitrectomy specimens [5–7]. However, in most cases, detection of larvae in the eyes prior to enucleation is almost impossible.

Currently, the diagnosis of ocular toxocariasis is based on characteristic clinical findings and the results of immunodiagnostic tests. The most reliable and common diagnostic test for visceral toxocariasis is enzyme-linked immunosorbent assay (ELISA) of the serum by using the excretory–secretory antigen derived from the second-stage larvae of *T. canis* (TES) because of the high sensitivity and specificity of this assay [16,17]. However, the sensitivity of serological tests for detecting ocular toxocariasis is low [18], and

Abbreviations: Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; OD, optical density; TES, excretory–secretory antigen isolated from second-stage larvae of *Toxocara canis*; PBS, phosphate-buffered saline; PBS/T, PBS containing 0.05% Tween 20; BSA, bovine serum albumin; BSA-PBS/T, PBS/T containing 0.2% BSA; HRP, horseradish peroxidase.

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several cases have been reported in which the serum titers of antibodies against *T. canis*, as determined using ELISA, were low, though larvae were detected in the enucleated eyes [19,20].

Some patients with suspected ocular toxocariasis were found to be negative for antibodies against *T. canis* or have low titers in the serum and have high titers in the intraocular fluid [8,9,21–24]. Thus, intraocular fluid is considered to have greater diagnostic accuracy than serum. However, little is known about the intraocular antibody profile in humans and experimentally infected animals. The factors that need to be elucidated are (a) the timing of the increase in the intraocular level of antibodies against *T. canis* after the onset of infection, (b) the duration for which these antibody levels remain elevated, and (c) the correlation between intraocular antibody levels and the severity of intraocular inflammation. We investigated the parasite-specific antibody profile in the aqueous humor of rabbits infected with *T. canis* via the intravitreal and oral routes for precise diagnosis of ocular toxocariasis.

Antibodies to pathogens are found in the lacrimal fluid of patients with uveitis due to conditions such as cytomegalovirus retinitis and ocular toxoplasmosis, and the detection of such pathogen-specific antibodies in tears is reported to be useful for diagnosis [25,26]. In order to evaluate this new diagnostic specimen, we investigated the anti-*T. canis* immunoglobulin (Ig)G antibody profile in the tears of rabbits infected with *T. canis*.

2. Materials and methods

2.1. Animals

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. Six male pigmented Dutch rabbits were purchased from Biotek Co. Ltd. (Saga, Japan). At the time of infection with *T. canis*, the rabbits were 9–13 weeks old with a mean age of 11 weeks and weighed 1.4–1.8 kg with a mean weight of 1.5 kg.

2.2. Parasites

T. canis eggs were obtained from the uteri of adult female worms, and the worms were collected from the feces of naturally infected puppies following the administration of anthelmintics. Mature embryonated eggs were prepared according to the method of Oshima [27]. Second-stage larvae were collected aseptically and maintained in a culture medium. Prior to intravitreal injection, the larvae were repeatedly washed with the culture medium and then suspended in physiological saline.

2.3. Infection procedures

We randomly divided 6 rabbits into 2 groups: the intravitreally infected group (Vit group) and the orally infected group (Ora group) (Table 1). We also collected samples from each rabbit before infection; these samples were used as normal controls.

We intravitreally injected *T. canis* larvae into the right eye of 4 rabbits in the Vit group (Vit 1, 2, 3, and 4), even though this is an unnatural mode of infection. The rabbits were anesthetized with a subcutaneous injection of xylazine (1.0 mg/kg; Bayer HealthCare, Berlin, Germany) and an intramuscular injection of ketamine hydrochloride (23.0 mg/kg; Yell Pharmaceutical Co. Ltd., Tokyo, Japan). Their pupils were dilated with tropicamide and phenylephrine hydrochloride (Santen Pharmaceutical Co. Ltd., Osaka, Japan). Levofloxacin (Santen Pharmaceutical Co. Ltd.) and oxybuprocaine hydrochloride (Santen Pharmaceutical Co. Ltd.) were instilled into their right eyes. With a sterilized 30-gauge needle, approximately 20 live second-stage larvae were injected into the vitreous cavity through the sclera; the injection site was located 1.0–1.5 mm from the corneal limbus and over the pars plana. After injection, we confirmed the

Table 1

Experimental design for sampling and observing ocular and histopathological changes in rabbits infected with *Toxocara canis*.

Rabbits	Vit group (intravitreal infection)		Ora group (oral infection)	
	Vit 1 and Vit 2	Vit 3 and Vit 4	Ora 1	Ora 2
Parasites ^a	20 larvae	20 larvae	5500 eggs 30,000 eggs	1100 eggs 30,000 eggs
Time point for sampling ^b				
Serum	0–11	0–20	0–20 ^c	
Aqueous humor	0–11	0–20	0–19 ^c	
Vitreous humor	NP ^d	20	20	
Tears	NP ^d	0–20	0–20 ^c	
Ocular observation ^b	0–11	0–20	0–20	
Histopathological observation ^b	11	20	20	

^a We intravitreally injected approximately 20 live second-stage larvae of *T. canis* into the right eye of Vit 1, 2, 3, and 4. Ora 1 and 2 were orally administered 5500 and 1100 embryonated eggs of *T. canis*, respectively, and another 30,000 eggs were administered to these 2 rabbits at 4 weeks after the first administration.

^b “0–11”, for example, indicates that the sampling or observation was performed every week from 0 to 11 weeks after infection.

^c Not sampled at 4 weeks after infection.

^d NP: sampling was not performed.

presence of motile larvae in the vitreous cavity and the absence of trauma to the retina; we then instilled drops of levofloxacin into the eye. The left eye of each rabbit was intact.

Two rabbits in the Ora group, Ora 1 and 2, were administered 5500 and 1100 embryonated *T. canis* eggs, respectively, by gavage, under light anesthesia with ketamine hydrochloride (23.0 mg/kg); this route resembles the natural mode of infection. We performed the Ouchterlony test for serum antibodies against TES at 3 weeks after the administration of *T. canis* eggs. Since no antibodies were detected, another 30,000 eggs were administered to the 2 rabbits at 4 weeks after the first administration.

2.4. Blood, and aqueous and vitreous humor collection

We collected blood and aqueous humor samples every week, from week 0 (i.e., before the infection) to week 11 or 20 after the infection (Table 1). Approximately 3.0 ml of blood was collected from the marginal ear vein of each rabbit. To obtain aqueous humor samples, the rabbits were anesthetized with xylazine (1.0 mg/kg) and ketamine hydrochloride (23.0 mg/kg), and mydriatics, antibiotics, and topical anesthetics were instilled into their eyes. Approximately 100 µl of aqueous humor was obtained via limbal paracentesis with a sterilized 27-gauge needle attached to a tuberculin syringe. After each aqueous humor sample was collected, we instilled antibiotics and betamethasone sodium phosphate (Shionogi & Co. Ltd., Osaka, Japan) into the eyes of the rabbits 4 times on the same day. Finally, we sacrificed the rabbits, enucleated their eyes, and collected vitreous humor samples via the pupils. Blood, and aqueous and vitreous humor samples were centrifuged (3000 rpm for 10 min at 4 °C), and the supernatants were stored at –30 °C until use.

2.5. Tear collection

Every week, we collected tear samples from 2 rabbits in each group by using blood-sampling papers (Strip Type; Toyo Roshi Kaisha Ltd., Tokyo, Japan) (Table 1). We successively placed 2 pieces of the paper between the upper palpebral and bulbar conjunctivae of each eye for 1 min, without using topical anesthesia. After drying, the papers were stored at –30 °C until use. In order to perform ELISA on the collected tear samples, the papers were cut into several pieces and immersed overnight in 400 µl of phosphate-buffered saline (PBS) (pH, 7.4) at 4 °C.

2.6. ELISA protocol for assaying IgG, IgA, and IgM

Using ELISA, we measured the levels of IgG, IgA, and IgM against TES in the serum, and aqueous and vitreous humor samples and the level of IgG against TES in the tears. TES was prepared according to the protocol of de Savigny [28]. ELISA was carried out as reported by Matsuda et al. [29], with slight modifications. In brief, 96-well microtiter plates (Greiner Bio-One Co. Ltd., Seddanstrasse, Germany) were coated overnight with 1.0 µg protein/ml TES (100 µl/well) in bicarbonate buffer (pH, 9.5) at 4 °C [30]. The plates were washed 5 times with PBS containing 0.05% Tween 20 (PBS/T) (pH, 7.2) and then blocked with PBS containing 1.0% bovine serum albumin (BSA) for 2 h at room temperature. After the plates were washed, they were probed with rabbit serum, and aqueous and vitreous humor samples (dilutions, 1:100) in PBS/T containing 0.2% BSA (BSA-PBS/T) (100 µl/well) for 1 h at 37 °C. Tear samples in PBS were used without diluting them.

In order to measure IgG, we used horseradish peroxidase (HRP)-conjugated goat IgG fraction against rabbit IgG (MP Biomedicals Inc., Aurora, USA) diluted to a ratio of 1:3000 in BSA-PBS/T. The plates used for IgG measurement were washed and then incubated with 100 µl of the diluted HRP-conjugated anti-rabbit IgG for 1 h at 37 °C.

For IgA and IgM measurements, biotin-conjugated mouse anti-rabbit IgA (BD Biosciences, Franklin Lakes, USA) and biotin-conjugated mouse anti-rabbit IgM (BD Biosciences) were diluted to a ratio of 1:1000 and 1:3000 in BSA-PBS/T, respectively. The plates used for the measurements were washed, and 100 µl of the diluted anti-rabbit IgA or IgM was incubated for 1 h at 37 °C. Subsequently, the plates were washed again and then incubated for 1 h at 37 °C with streptavidin-HRP conjugate (BD Biosciences) diluted in BSA-PBS/T (1:1000 for the IgA assay and 1:3000 for the IgM assay).

In the case of IgG, IgA, and IgM assays, we dissolved 1 tablet of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (10 mg; Sigma, St. Louis, USA) in 10 ml of 0.05 M citrate buffer (pH, 5.0). Immediately before use, we added 100 µl of 0.3% H₂O₂ to this solution. The plates were washed again, and 100 µl of this substrate was added to each well for color development. After incubation for 30 min at room temperature, the optical density (OD) at 405 nm was measured using an automatic plate reader (Wallac 1420; PerkinElmer™ Life Sciences, Turku, Finland).

We tested each sample in triplicate and averaged the OD values (OD_{sample}). Three wells in each plate were used as blanks; i.e., the same procedure was used for these 3 wells, except that the rabbit samples were not added. The OD of each sample was calculated using the following formula: OD = OD_{sample} – OD_{blank}.

2.7. ELISA protocol for assaying IgE

The ELISA protocol for assaying IgE against TES in the serum, and aqueous and vitreous humor samples was identical to that for IgG, IgA, and IgM. However, for the measurement of IgE, the samples were

diluted to a ratio of 1:10 and were incubated for 2 h for better color development. Since anti-rabbit IgE antibody was not available, we used rat anti-human IgE conjugated with HRP (Experimental Immunology Unit, France) diluted to 1:1000. According to the product information, this reagent could react with both human and rabbit IgE.

2.8. Ophthalmologic examination

We observed the anterior segment through a loupe (magnification, ×2.3) every day and carried out ophthalmoscopic examinations every week in order to determine whether intraocular antibody formation and intraocular inflammation were correlated (Table 1). We used a specially adapted ophthalmoscope for observing the ocular fundi of small animals (VMS-170S; Scleral Corporation, Tokyo, Japan); examination with this instrument requires that the probe of the ophthalmoscope be brought into contact with the cornea. Prior to the ophthalmoscopic examinations, we induced mydriasis and then general anesthesia with xylazine and ketamine hydrochloride injection and topical anesthetics. We also instilled a drop of hydroxyethylcellulose (Senju Pharmaceutical Co. Ltd., Osaka, Japan) into the eyes of the rabbits. The images of the fundi were transferred to a TV monitor and videotaped.

2.9. Histopathological examination

All animals were sacrificed at the end of the experiment by the administration of pentobarbital sodium (Schering-Plough Animal Health K.K., Tokyo, Japan) (Table 1). The enucleated eyeballs were fixed in a solution containing 4.0% glutaraldehyde, 10% neutral formalin, and 0.03 M phosphate buffer (pH, 7.2) in a ratio of 1:1:2 by volume. The eyeballs were then embedded in JB-4 plastic resin (Polysciences Inc., Warrington, UK), sectioned serially at 1.5 µm intervals, and then stained with May–Grünwald–Giemsa stain for microscopic examinations.

3. Results

3.1. Ophthalmologic examination

The ophthalmoscopic findings are summarized in Table 2. No particular ocular findings were observed in the left eyes of Vit 1, 2, 3, and 4 and both eyes of Ora 2. We classified the severity of vitreous opacity as negative, slight, mild, and severe. Severe vitreous opacity was diagnosed when the fundus was scarcely visible on ophthalmoscopic examination. When the fundus could be seen almost entirely, the severity of the vitreous opacity was classified as slight.

A vitreous opacity and cataract developed in the right eye of Vit 1 (Fig. 1A and B). We consider that severe intraocular inflammation led to cataract formation (complicated cataract). Retinal hemorrhages in the region of myelinated nerve fibers, a vitreous opacity, and an iris

Table 2
Ophthalmoscopic findings in rabbits infected with *Toxocara canis* eggs or larvae.

Eyes of rabbits ^a	Vit group				Ora group	
	RE of Vit 1	RE of Vit 2	RE of Vit 3	RE of Vit 4	RE of Ora 1	LE of Ora 1
Larvae in the vitreous cavity ^b	0	0–4, (6)	0–20	1, 7, and 19	Negative	Negative
Severity of vitreous opacity ^c	Severe: 1–4 ^d	Mild: 1–4 Severe: 5–11	Mild: 1–4 Slight: 5–8 Negative: 9–20	Mild: 1 Severe: 2–20	Negative: 1–5 Mild: 6–20	Negative: 1–20
Retinal hemorrhages ^c	Negative	1	Negative	Negative	5	7
Other findings ^c	Cataract: 4–11 ^d	Iris nodule: 7–11	Negative	Negative	Negative	Negative

^a RE, right eye and LE, left eye.

^b “0” indicates that we observed larvae in the vitreous cavity right after infection. “0–4” indicates that we observed larvae in the vitreous cavity from 0 to 4 weeks after infection. “Negative” indicates that we did not observe larvae throughout the period of our experiment. “(6)” indicates that a larva was detected in the anterior chamber at 6 weeks after infection.

^c “1–4”, for example, indicates that ophthalmoscopic findings were observed from 1 to 4 weeks after infection.

^d It was not possible to conduct an ophthalmoscopic examination of the right eye of Vit 1 from 5 weeks after infection because of the development of mature cataract.

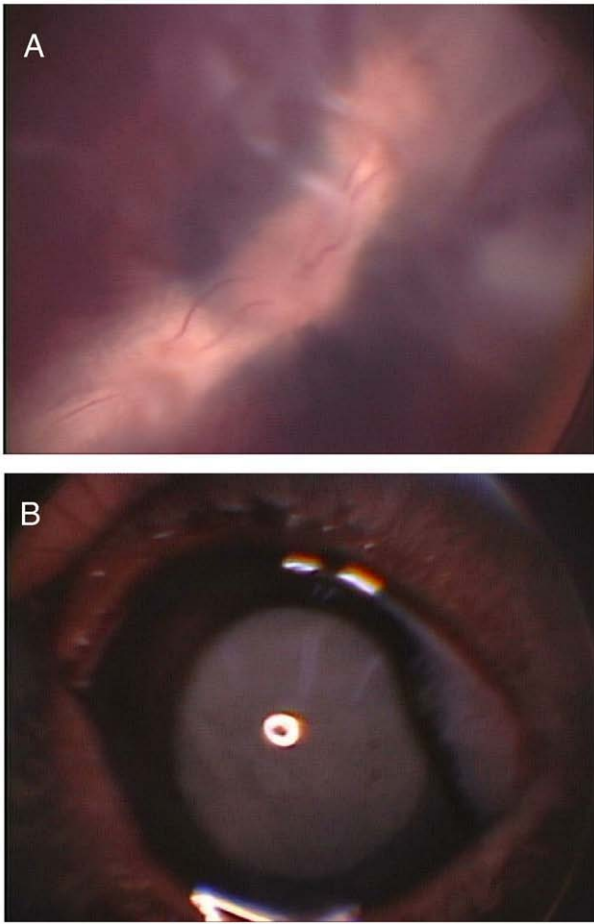


Fig. 1. The right eye of Vit 1. (A) Severe vitreous opacity at 1 week after infection. (B) Mature cataract at 9 weeks after infection.

nodule were observed in the right eye of Vit 2 (Fig. 2). The vitreous opacity in the right eye of Vit 3 almost disappeared at 9 weeks after infection (Fig. 3A and B). Vit 4 exhibited a vitreous opacity in its right eye. Ora 1 showed a vitreous opacity and we observed small, localized retinal hemorrhages (1) in the region of myelinated nerve fibers, (2) on the optic disc, and (3) in other areas of the retina of the right eye (Fig. 4A and B). We also observed small retinal hemorrhages in the region of myelinated nerve fibers in the left eye (Fig. 5).

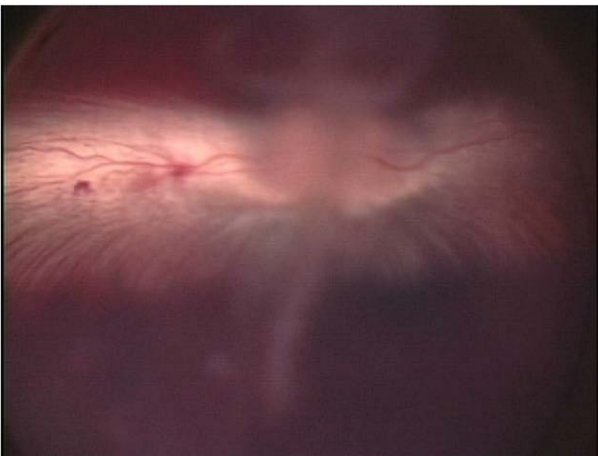


Fig. 2. The right eye of Vit 2. Retinal hemorrhages and mild vitreous opacity at 1 week after infection.

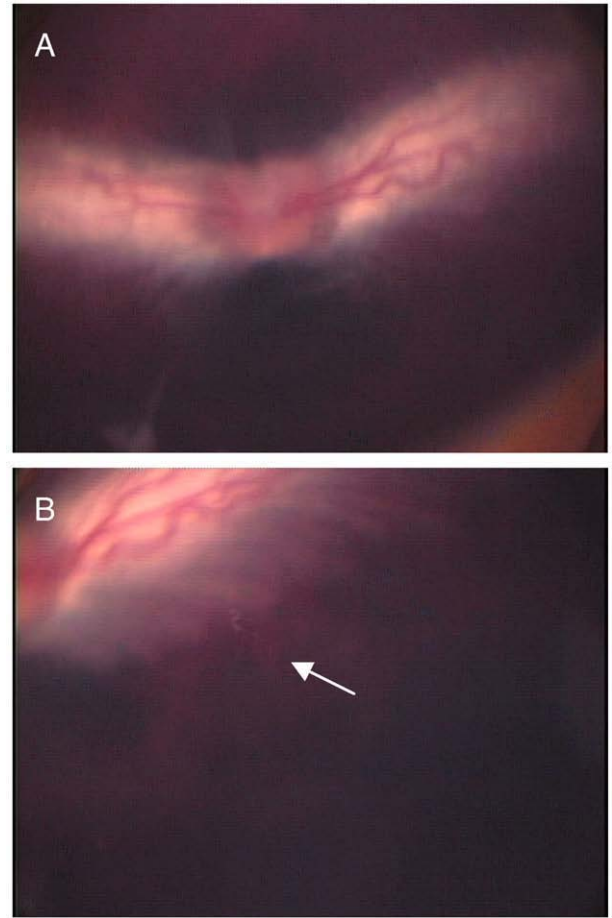


Fig. 3. The right eye of Vit 3. (A) Mild vitreous opacity at 4 weeks after infection. (B) Slight vitreous opacity at 7 weeks after infection. Slow-moving *T. canis* larvae were observed in the vitreous cavity. The arrow indicates a larva in the vitreous cavity.

At 20 weeks after infection, 2 or 3 larvae were detected in the vitreous cavity of the right eye of Vit 3. Initially, the injected larvae were actively motile, but after 1 week, they moved very slowly.

3.2. Histopathological examination

A larva was detected in the rod and cone layer of the retina of the right eye of Vit 1 (Fig. 6). Leukocytic infiltrates were present around the larval fragments. In our study, no other histopathological ocular changes were observed in any of the rabbits.

3.3. Anti-TES IgG

With regard to the IgG assay, all rabbits in both groups showed similar changes in the ODs of the serum samples; the serum OD started to increase from 2–3 weeks after infection, peaked at 5–11 weeks after infection, and remained elevated until the end of the experiment (Fig. 7A).

The ODs of the aqueous humor samples collected from the right eye of the Vit group rabbits exceeded 0.1 from 2–4 weeks after infection and peaked at 7–15 weeks after infection; in Vit 1, 2, and 4, the ODs remained >0.1 until the end of the experiment (Fig. 7B). The ODs of the aqueous humor samples collected from the left eye of Vit 1 and 2 increased slightly, but those of the aqueous humor samples collected from the left eye of Vit 3 and 4 did not increase (Fig. 7C). The ODs of the aqueous humor samples collected from both eyes of the Ora group rabbits slightly increased from approximately 5 weeks after infection (Fig. 7B and C).

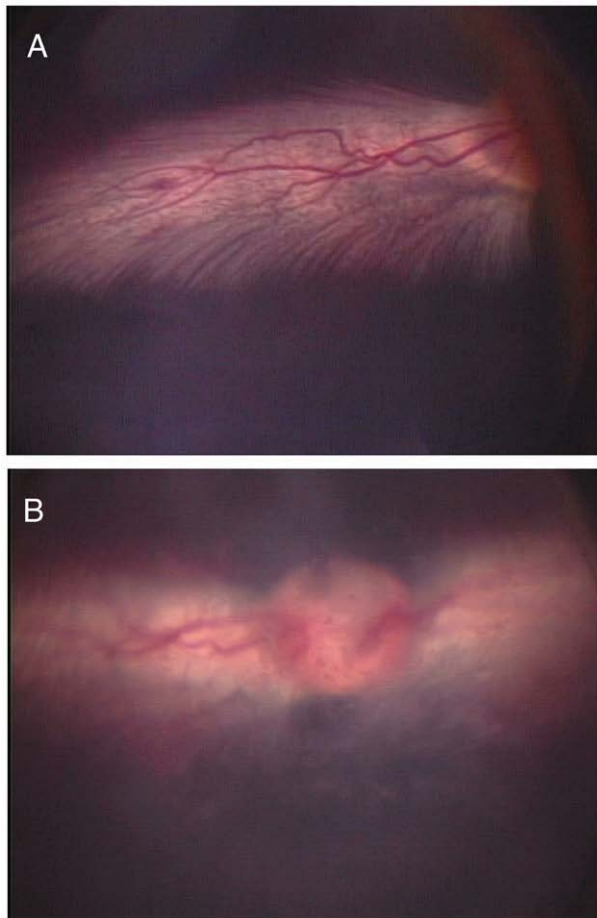


Fig. 4. The right eye of Ora 1. (A) Small, localized retinal hemorrhages were observed in the region of myelinated nerve fibers at 5 weeks after infection. (B) Mild vitreous opacity was observed at 20 weeks after infection.

The vitreous humor sample collected from the left eye of Vit 3 was found to contain many erythrocytes on microscopic examination and was therefore excluded from analysis. The ODs of the other vitreous humor samples nearly equaled those of the aqueous humor samples collected at 19 or 20 weeks after infection (Fig. 7B and C).

The ODs of the tear samples used for the IgG assay began to increase from 2–7 weeks after infection, and thereafter, a moderate

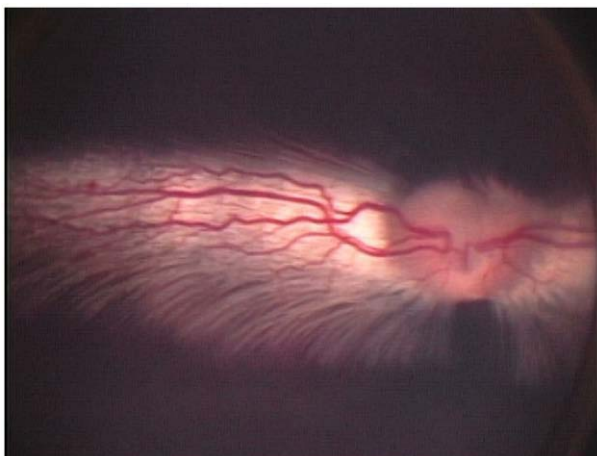


Fig. 5. The left eye of Ora 1. Small retinal hemorrhages in the region of myelinated nerve fibers at 7 weeks after infection.

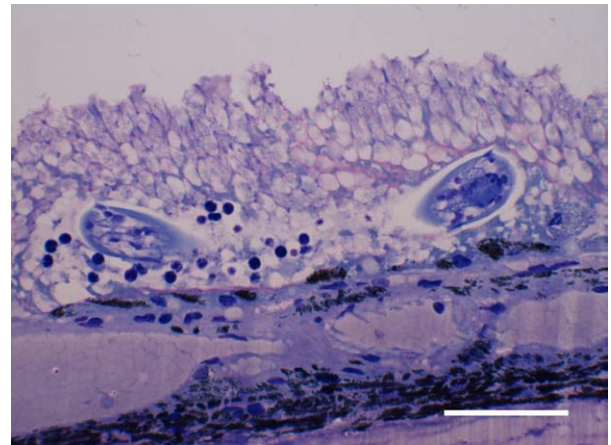


Fig. 6. Light microscopic examination of the right eye of Vit 1 at 11 weeks after infection. Larval fragments were detected in the cone and rod layer of the retina. Infiltrations of leukocytes around the larval fragments were observed, but no other particular inflammatory changes were observed in the retina (May–Grünwald–Giemsa staining; magnification, $\times 400$; scale bar, 50 μm).

increase was detected in the case of most samples from both groups. The ODs of the tear samples were lower than those of the serum samples (Fig. 8A,B, and C).

3.4. Anti-TES IgA

No significant increase in the ODs of the serum samples used for the IgA assay was detected in both groups (Fig. 9A). Only the aqueous humor samples collected from the right eye of Vit 1, 2, and 4 showed increased ODs. The ODs of these aqueous humor samples increased at about 2–5 weeks after infection and then rapidly declined (Fig. 9B and C). The ODs of the vitreous humor samples did not increase (Fig. 9B and C).

3.5. Anti-TES IgM

All rabbits in both groups showed similar changes in the ODs of the serum samples used for the IgM assays. The serum ODs began to increase from 2–3 weeks after infection, peaked at 3–6 weeks after infection, rapidly decreased thereafter, and then remained low (Fig. 10A).

In the case of the aqueous humor samples, only the samples collected from the right eye of the Vit group rabbits showed increased ODs. This increase in the ODs of the aqueous humor samples was not pronounced; it was first observed at 2–4 weeks after infection and lasted for several weeks (Fig. 10B and C). The ODs of the vitreous samples did not increase (Fig. 10B and C).

3.6. Anti-TES IgE

No significant increase was detected in the ODs of the serum, and aqueous and vitreous humor samples used for the IgE assay in both groups (data not shown).

4. Discussion

Takayanagi et al. reported that when orally infected with *T. canis* eggs, Mongolian gerbils show a high incidence of ocular toxocariasis but mice, rabbits, guinea pigs, and monkeys show a low incidence of ocular toxocariasis [31]. However, we found that the aspiration of aqueous humor from the eye of a Mongolian gerbil was very difficult because the eyeball was small in size. Dzbeński et al. also reported that ocular inflammation was not observed in rabbits that were orally infected with *T. canis* eggs [32], but the aqueous humor can be aspirated from the eyes of rabbits repeatedly and safely. Therefore, we used rabbits in our study.

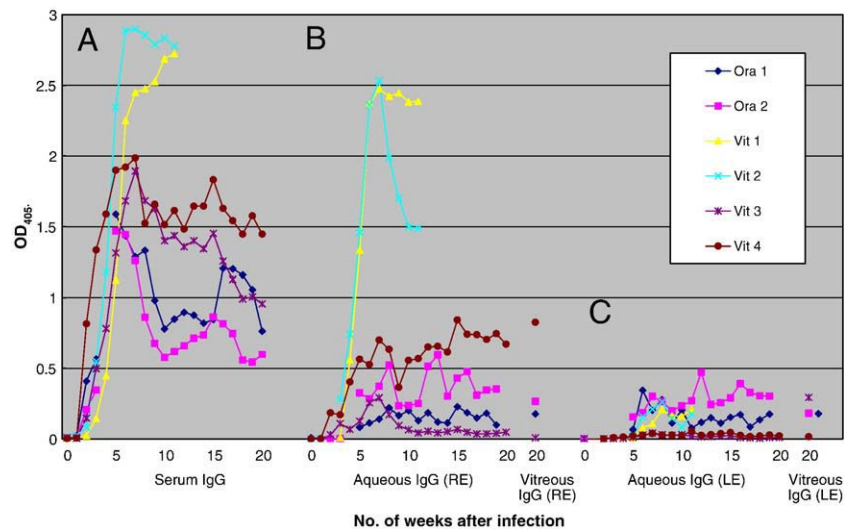


Fig. 7. ELISA profiles of anti-*Toxocara canis* IgG antibodies in the serum, and aqueous and vitreous humor of rabbits infected with *T. canis* eggs (Ora group) or larvae (Vit group). (A) Serum IgG, (B) IgG in the aqueous and vitreous humor of the right eye (RE), and (C) IgG in the aqueous and vitreous humor of the left eye (LE).

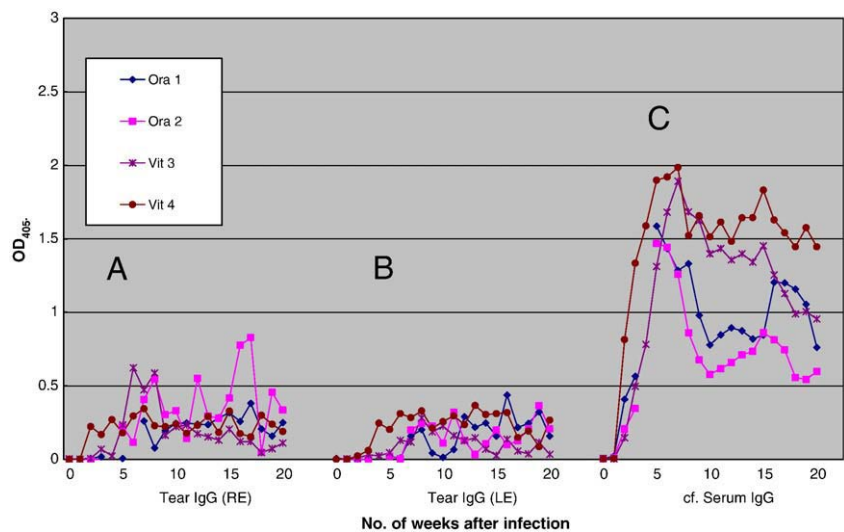


Fig. 8. ELISA profiles of anti-*Toxocara canis* IgG antibodies in the tears of rabbits infected with *T. canis* eggs (Ora group) or larvae (Vit group). (A) IgG in the tears of the right eye (RE), (B) IgG in the tears of the left eye (LE), and (C) serum IgG (reference).

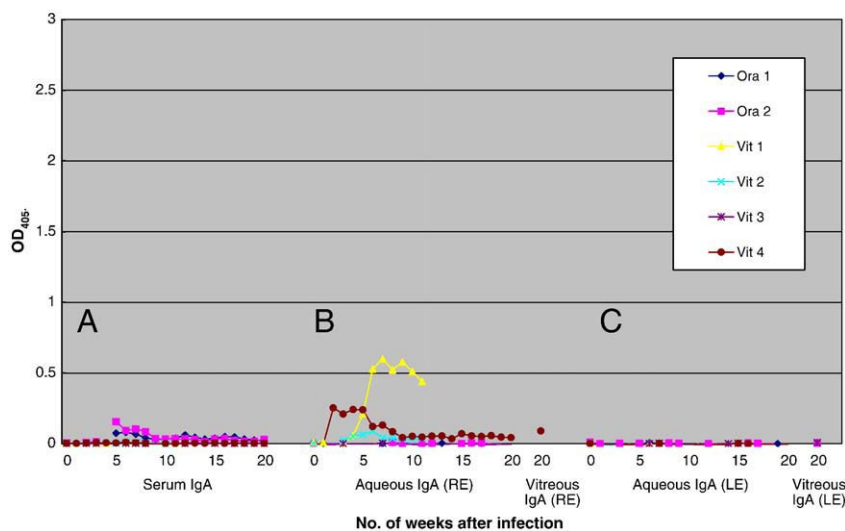


Fig. 9. ELISA profiles of anti-*Toxocara canis* IgA antibodies in the serum, and aqueous and vitreous humor of rabbits infected with *T. canis* eggs (Ora group) or larvae (Vit group). (A) Serum IgA, (B) IgA in the aqueous and vitreous humor of the right eye (RE), and (C) IgA in the aqueous and vitreous humor of the left eye (LE).

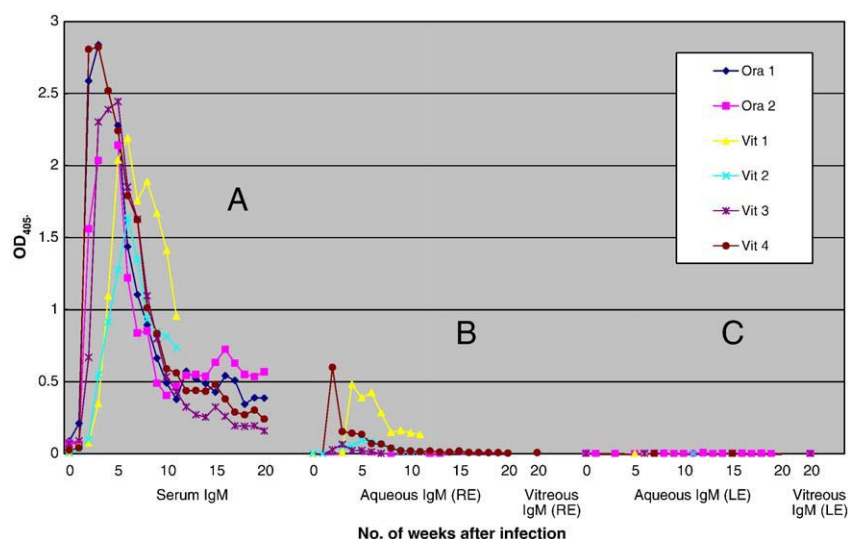


Fig. 10. ELISA profiles of anti-*Toxocara canis* IgM antibodies in the serum, and aqueous and vitreous humor of rabbits infected with *T. canis* eggs (Ora group) or larvae (Vit group). (A) Serum IgM, (B) IgM in the aqueous and vitreous humor of the right eye (RE), and (C) IgM in the aqueous and vitreous humor of the left eye (LE).

Watzke et al. reported the injection of *T. canis* larvae in cynomolgus monkeys through the intravitreal, periocular, systemic, and intracarotid routes [33]; they also reported that injecting *T. canis* larvae via the intravitreal route was the best method for inducing ocular toxocariasis [34]. Therefore, in this study, we infected rabbits with *T. canis* via the intravitreal route and the usual oral route.

In this study, we analyzed the intraocular antibody profile in an animal model of ocular toxocariasis in order to develop a more precise method for the diagnosis of ocular toxocariasis.

Rabbits that have been orally infected with *T. canis* eggs reportedly exhibit parasite-specific antibodies in the serum at 2–4 weeks after the infection; the antibody level peaks at 3–6 weeks after infection and remains high for a long time [35,36]. In this study, the ODs of the serum samples collected from the Ora and Vit group rabbits and used for the IgG assays corresponded to the ODs reported in previous studies, regardless of the presence of intraocular inflammation. This finding suggests that ELISA has low specificity for the analysis of serum samples for the diagnosis of ocular toxocariasis.

The immunodiagnostic significance of intraocular fluid has not yet been described in sufficient detail. Thus far, little is known about the time course of the changes in the intraocular antibody profile in ocular toxocariasis. Dzbeński et al. orally infected rabbits with *T. canis* eggs; collected aqueous humor at 1, 3, and 6 months after infection; and detected anti-*T. canis* IgG in the collected aqueous humor samples [32]. To our knowledge, this is the only report in which intraocular antibodies were assayed repeatedly. In the present study, the severity of the vitreous opacity in the right eye of Vit 1, 2, 3, and 4 and the ODs of the aqueous humor samples collected from these rabbits and used for the IgG assay seemed to change concordantly. The vitreous opacity in the right eye of Vit 3 nearly disappeared at 9 weeks after infection, and the OD of the aqueous humor samples collected from this rabbit peaked at 7 weeks after infection and decreased to <0.1 at 9 weeks after infection. On the basis of the intraocular antibody profiles of Vit 1, 2, 3, and 4, we suggest that the IgG level in the aqueous humor reflects intraocular inflammation and that the appropriate time for sampling the aqueous humor is at least 4 weeks after the onset of intraocular inflammation.

The IgA and IgM levels in the aqueous humor also seemed to correspond to the presence of intraocular inflammation, but the ODs for the IgA and IgM assays were low. Therefore, we do not consider the IgA and IgM levels in the aqueous humor to be of diagnostic value.

The ODs of the vitreous humor samples used for the IgG assay were similar to the ODs of the aqueous humor samples that were collected almost simultaneously. Therefore, we believe that aqueous humor

samples can be used instead of vitreous humor samples for immunological tests.

It should be noted that although no inflammatory reactions were noted in the left eye of Vit 1 and 2 and in both eyes of Ora 2, small increases were detected in the ODs of the aqueous humor samples used for the IgG assays. Thus, the ODs of intraocular fluids may increase in the absence of intraocular inflammation or larvae.

Weekly paracentesis might have resulted in the breakdown of the blood–aqueous barrier. In the case of the IgG assays, the ODs of the serum samples from the Vit group increased, and the ODs of the aqueous humor samples collected from the left eyes of Vit 1 and 2, which exhibited neither intraocular inflammation nor intraocular larvae, also increased. These results can be explained by the diffusion of parasite-specific antibodies through a leaky blood–aqueous barrier. In the Ora group, the ODs of the serum samples used for the IgG assays increased earlier than the ODs of the aqueous humor samples used for the same assay. In the Vit group, in the case of the IgG assays, the ODs of the aqueous humor samples collected from the right eye and the serum samples increased earlier than the ODs of the aqueous humor samples collected from the left eye. These time lags may be due to antibody diffusion through a defective blood–aqueous barrier. When the barrier breakdown is significant, the ODs of all samples will eventually become equal. Thus, the apparent discordance between serum IgG and aqueous humor IgG levels cannot be explained solely on the basis of the disruption of the blood–aqueous barrier (Fig. 7A,B, and C). We suppose that this discordance in the IgG levels reflects local antibody production in the eye; however, we did not determine the Goldmann–Witmer coefficient for the possibility of local antibody production [21,37,38].

Table 3

Maximum ODs of IgG antibodies, as measured by ELISA, against TES in the serum and aqueous humor of rabbits infected with *Toxocara canis* larvae or eggs.

Rabbits	Vit group				Ora group	
	Vit 1	Vit 2	Vit 3	Vit 4	Ora 1	Ora 2
Serum IgG	2.72	2.90	1.89	1.99	1.59	1.47
RE ^a : IgG in the aqueous humor	2.47	2.53	0.29	0.84	0.23	0.59
LE ^b : IgG in the aqueous humor	0.21	0.26	0.04	0.05	0.34	0.47

^a RE, right eye.

^b LE, left eye.

The cutoff antibody level for the diagnosis of human ocular toxocariasis has not yet been standardized, and different laboratories use different cutoffs [39]. The maximum ODs of IgG antibodies against TES in the serum and aqueous humor samples of each rabbit are shown in Table 3. We observed motile larvae in the vitreous cavity of the right eye of Vit 3 every week, from just after injection of larvae until 20 weeks after infection; however, the maximum OD of aqueous humor IgG antibodies was as low as 0.29. This suggests that the absence of intraocular anti-*T. canis* antibodies does not always rule out the presence of intraocular larvae and that the timing of collection of intraocular fluids is important in the diagnosis of ocular toxocariasis.

The ELISA test for ocular toxocariasis has some drawbacks. The ELISA protocols and diagnostic cutoffs are not standardized [39]. Not all studies have reported the cutoffs that were considered to denote a positive result, and the starting dilutions of specimens vary across laboratories [40]. Further, it would be ethically inappropriate to collect intraocular fluid from healthy people for use as normal controls. These drawbacks might explain why some patients with suspected ocular toxocariasis have negative or low titers in the serum and positive or high titers in the intraocular fluid on ELISA. Moreover, the time at which blood and intraocular fluids are sampled might also be responsible for the above finding.

In both the Vit and Ora groups, we detected anti-*T. canis* IgG in tears, but the tear IgG levels did not correspond to the severity of intraocular inflammation. Hence, we suppose that the anti-*T. canis* antibodies found in the tears originated in the systemic circulation. Thus, in this study, the antibody levels in the tear samples could not be used to diagnose ocular toxocariasis; however, this specimen will probably have clinical use, provided the various steps involved in assay procedures, such as tear collection, are improved.

The doses of the injected *T. canis* larvae and eggs were determined by referring to previous studies [32,35,36]. The Vit group (4 rabbits) received approximately 20 larvae, whereas the Ora group (2 rabbits) received 5500 and 1100 eggs and were then challenged with 30,000 eggs. The low dose of local infection and high dose of systemic infection resulted in similar antibody production profiles of the serum IgG and IgM and tear IgG. We were unable to determine the reason for this phenomenon in our present study, but the mechanism of local immune response of the eye may be different from that of other local responses. Further studies are required to understand this issue.

Recently, specific IgE antibodies have been reported to be useful for the diagnosis of ocular toxocariasis [41,42]. However, in our study, no significant increase was detected in the IgE levels of the serum, and aqueous and vitreous humor samples in both groups. We think that this was because of the materials and methods used by us. Further studies are required to elucidate the importance of IgE antibodies as a marker of ocular toxocariasis.

Histopathological studies have been conducted on the eyes of laboratory animals infected with *T. canis*. Mongolian gerbils [31,43], guinea pigs [44], and mice [45] were infected with *T. canis* eggs orally, and cynomolgus monkeys [33] were infected with *T. canis* larvae intravitreally. Histopathological findings such as eosinophil infiltration were obtained for all the animals. However, no consensus was reached on the retinal damage in these animals. In the present study, regardless of the ophthalmoscopic findings, the retinas of the rabbits were not significantly injured. Moreover, infiltration of leukocytes around larval fragments was detected in the retina of the right eye of Vit 1, but no other particular inflammatory changes were observed in the retina. We consider that *T. canis* larvae do not directly damage the rabbit eye, and other factors seem to be required for the development of retinal lesions such as granuloma.

We investigated the parasite-specific antibody profile in the aqueous humor of rabbits infected with *T. canis* and confirmed that the anti-*T. canis* IgG levels in the aqueous humor are well correlated with the severity of intraocular inflammation. In addition, we

determined that the appropriate time for aqueous humor collection is at least 4 weeks after the onset of intraocular inflammation. Our findings may be useful in assisting the immunological diagnosis of human ocular toxocariasis. Further studies are required to determine whether our results are applicable to humans.

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