Experimental Ocular Toxocariasis in Mice Infected with Long-term-maintained Embryonated Eggs of Toxocara canis

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Ocular injury to ICR mice from embryonated eggs of Toxocara canis, which had been maintained in 2% formalin for 14 months at 4°C, was evaluated by microscopic and pathologic assessments at 1, 2, 3, 5, 7, 28, 56, 84, 112, 140, 168, 196, 294 and 469 days post infection (DPI). On each date, three infected mice and two age-matched uninfected control mice were sacrificed; left eyeballs from infected mice were examined for larvae under a dissecting microscope while all right eyeballs were embedded in paraffin for histologic study. No larvae were observed in the left eyeball of any of the 42 infected mice, while pathologic changes were observed in right eyeballs. Pathologically, the predominant changes were retinal detachment, iridocyclitis, and pars planitis, followed by optic nerve papillitis and superficial infarcts, as observed in one of three infected mice per group at 56, 140, and 168 DPI, respectively. No infiltrating cells surrounding the larvae in the retina were observed in the infected mice. This is the first report that larvae hatched from T. canis embryonated eggs maintained for a long time can cause murine ocular toxocariasis. These results further extend our knowledge of the pathogenicity of T. canis embryonated eggs.

Key Words: embryonated eggs, ICR mice, ocular toxocariasis, Toxocara canis

Toxocara canis, a cosmopolitan parasite of canines and the major agent of human toxocariasis, constitutes a serious epidemiologic problem in many countries. Worldwide surveys show that the prevalence of Toxocara infection ranges from 86% to 100% in pups and 3% to 81% in adult dogs [1]. Children and adults are infected by accidental ingestion of T. canis embryonated eggs. The major clinical consequences of prolonged migration of T. canis larvae in humans are visceral larva migrans (VLM) and ocular toxocariasis (OT) [2].

Many cases of OT in children or adults clinically associated with visual loss, pars planitis, endophthalmitis, choroidoretinitis, uveitis, and retinal detachment have been reported over the last 10 years [3–9]. However, the age of the larvae invading the eyes of infected patients, and the duration of the larvae that retain infectivity and pathogenicity to the eyes of human or paratenic hosts, remain unclear.

Oshima, using a larval recovery technique, observed that the infectivity of T. canis embryonated eggs decreased significantly when egg cultures were kept at room temperature for more than 2.5 months [10]. Thereafter, parasitologists worldwide studying experimental toxocariasis have generally followed his advice of using embryonated eggs kept for not more than 3 months at room temperature [11,12]. Further studies also supported his advice, indicating that decreased viability and infectivity were related to changes in the biologic properties of larvae during long-term incubation of at least 5 to 11 months at room temperature in vitro and in vivo [13–16]. Nevertheless, in recent years, fertilized eggs cultured to reach embryonated status have been stored at 4°C until use [17–20].
Recently, we confirmed that larvae hatched from *T. canis* embryonated eggs experimentally cultured in 2% formalin for at least 14 months at 4°C *in vitro* were capable of eliciting a Th2-dominant VLM [21]. However, our previous effort was mainly centered on experimental VLM. It is not known whether hatched larvae from *T. canis* eggs maintained for a long time are also able to invade the eyes and cause OT.

This study investigated whether larvae were able to invade the eyes and cause OT, as determined by larval recovery and pathologic assessments in mice infected with *T. canis* embryonated eggs maintained for 14 months.

**MATERIALS AND METHODS**

**Egg culture and the inoculation protocol**
Egg culture and the inoculation protocol have been described previously [21]. Briefly, adult *T. canis* worms were collected from the intestines of necropsied stray dogs [22], and infective eggs were cultured according to the method of Bowman et al with slight modifications [23]. Female worms were dissected and the anterior third of the uterus was stirred in 10 mL of a 1% sodium hypochlorite solution and incubated for 5 minutes at room temperature. Thereafter, distilled water was added to bring the volume up to 20 mL. The mixture was then filtered through two layers of mesh to remove large tissue debris, and the resulting material was centrifuged for 5 minutes at 2,000g. The supernatant was discarded and the pellet was washed twice with distilled water and once with 2% formalin. The eggs were resuspended in 2% formalin and placed in a 250 mL Erlenmeyer flask; 2% formalin was added to bring the liquid level to approximately 1 cm deep. The flask was covered with paraffin and incubated at room temperature (18–23°C) for 8 to 9 weeks with gentle weekly agitation. Thereafter, eggs were stored at 4°C for 14 to 15 months. They were washed with water to remove the formalin before use.

Female ICR mice aged 6 to 8 weeks were obtained from the Center for Experimental Animals, Academia Sinica, Taipei, Taiwan. Mice were housed in the animal facility of Taipei Medical University and maintained on commercial pelleted food and water *ad libitum*. Viability of the *T. canis* embryonated eggs maintained for 14 months was assessed using a light stimulation method before use [16]. Each mouse was infected with about 250 *T. canis* embryonated eggs in 100 µL of water by oral intubation.

**Microscopic examination**
A microscopic study was performed according to the method described by Olson with modifications [24]. Mice infected with the *T. canis* embryonated eggs were deeply anesthetized with ether and killed by heart puncture at 1, 2, 3, 5, 7, 28, 56, 84, 112, 140, 168, 196, 294, or 469 days post infection (DPI). On each date, three infected mice and two age-matched uninfected control mice were sacrificed and both eyes of each mouse were immediately removed. The left eyeball of each inoculated mouse was individually dissected by making an incision through the cornea. The fluid, lens, and soft inner tissues were scooped out onto a glass slide and examined for larvae under a dissecting microscope (Olympus, Ciba, Japan) at appropriate magnification. All animal experiments were carried out in accordance with institutional Policies and Guidelines for the Care and Use of Laboratory Animals and all efforts were made to minimize animal suffering.

**Histopathologic assessments**
A pathologic study was performed according to the method described in our previous study [21]. The right eyeball of each infected and age-matched uninfected control mouse was fixed separately in 10% neutral buffered formalin for at least 24 hours and embedded in paraffin. Several serial 5 µm sections were cut for each mouse. Paraffin was removed by heating the sections for 1 hour at 65°C. These sections were dehydrated by washing with xylene three times for 5 minutes each, then rehydrated through 100%, 95%, and 80% ethanol for 5 minutes each, and finally rinsed with distilled water. After staining with hematoxylin and eosin, pathologic changes were examined under a light microscope.

**RESULTS**
No larvae were observed in the left eyeball of any of the 42 infected mice, while pathologic changes were occasionally observed in some mice. Ocular pathologic changes were observed mainly in one of the three infected mice per group at 56, 140, and 168 DPI. Figure 1 shows the normal ocular anatomic structure of uninfected control mice, with a normal iris, retina, and optic nerve. Ocular pathologic changes in infected mice included retinal detachment (Figures 2 and 3), iridocyclitis and pars planitis (Figures 2–4), and optic nerve papillitis (Figures 3 and 5) at 56, 140, and 168 DPI. In addition to these four lesions, superficial infarcts were also observed at 168 DPI (Figure 6). The infiltrating cells in iridocyclitis and pars planitis were mainly lymphocytes and eosinophils. The larvae were located in the space between the detached retina and choroids at 56 DPI (Figure 2).
In contrast, larvae appeared outside (Figure 3) and within (Figure 5) the retina with apparent edema at 140 and 168 DPI, respectively. However, no inflammatory infiltration surrounding the larvae in the retina was observed in any of the infected mice (Figures 2, 3, and 5).

**DISCUSSION**

In the present study, although no larvae were observed in any of the left eyeballs of infected mice, larvae hatched from *T. canis* embryonated eggs maintained for a long time (14 months) were able to invade and elicit pathologic lesions in the right eyeballs of outbred ICR mice as monitored by pathologic study over 469 days. Our findings show that unilateral ocular invasion can be produced by one single larva. Nevertheless, the presence of more than one larva per eye observed in a murine model has occasionally been reported [25]. It seems that larval invasion into eyeballs is merely a random event related to probability. Ghafoor et al observed that larvae could be found in right, left, or both eyeballs of an inbred strain of infected C57BL mice when
infected with doses as high as 1,500 infective eggs during a 56-day investigation [26]. Whether this is related to the age and quantity of infective eggs used and utilization of different murine strains warrants further investigation.

On the other hand, it is possible that invasion into eyes is a consequence of larval migration towards the ocular chamber after a previous infection in another location [25]. Our previous study suggests that larvae hatched from T. canis embryonated eggs maintained for 14 months may be observed in various visceral organs including the liver, lungs, musculature, and brain of infected mice before 56 DPI [21].

The predominant ocular pathologic changes in outbred ICR mice infected with T. canis embryonated eggs maintained for 14 months were retinal detachment, iridocyclitis, and pars planitis, followed by papillitis and superficial infarcts. Most of these changes were also reported by Ghafoor et al, who found that the predominant histopathologic features in OT of inbred C57BL mice were iridocyclitis, pars planitis, and superficial microinfarcts followed by papillitis [26]. Inbred BALB/c mice do not seem to be an appropriate murine model for the study of OT because ocular pathologic lesions are rare [27]. It seems that outbred ICR mice are adequate for an OT study because this strain showed varying degrees of ocular pathologic changes in the trial. Only infected ICR mice, but not other murine models including gerbils, which were recently reported to be superior in experimental OT study [27–30], show retinal detachment, which is frequently found in human OT. Moreover, T. canis larvae maintained for 14 months may invade the eyes through the cerebrospinal fluid to the choroids and then to the retina. This is indirectly supported by the fact that larvae were identified in the choroids and that they induced retinal detachment at 56 DPI, and they subsequently penetrated the retina by 140 and 168 DPI. Several studies also propose that the most common route of invasion into eyes might be through the brain to the cerebrospinal fluid and then to the choroids [25,31].

It is unclear why no inflammatory infiltration surrounded the T. canis larvae in any of the infected mice at 56, 140, and 168 DPI. It might be proposed that the single larva invading the eyes was still alive because of its intact morphology [24], and a very mild inflammatory response was observed due to small amounts of larval antigens released into the eyes. It has been well documented that the pattern of deposition and sequestration of larval antigens in tissues is related to the variety of lesions produced in toxocariasis. The deposition of small amounts of larval antigens over a large area is likely to produce a diffuse low-grade inflammatory response, such as diffuse chronic endophthalmitis, whereas the deposition of large amounts of larval antigens locally is likely to produce an intense inflammatory response, such as a solitary granuloma [31]. Another explanation is that aged larvae seemed to retain the ability to rapidly shed their antigens, thus avoiding host immunologic damage in the eyes. Smith argues that rapid shedding of epicuticular antigens or surface-bound antibodies by larvae constitutes a mechanism for evading both antibody-dependent and antibody-mediated cellular cytotoxicity in vivo [31].

In conclusion, OT can occur concurrently with VLM in outbred ICR mice infected with T. canis infective eggs maintained for a long time (14 months) because hatched larvae were able to elicit pathologic lesions in the eyes as well as in the liver, lungs, brain, and musculature, as shown by our previous study [21]. This extends our knowledge of
the pathogenicity of *T. canis* embryonated eggs maintained in the long term.

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**REFERENCES**

小鼠感染長期培養的犬蛔蟲
受孕卵之實驗性眼球犬蛔蟲症

范家堃1 林永和2 洪健清3 蘇霏霖3
台北醫學大學 1院寄生蟲學科 2院病理學科
3院寄生蟲學科

本研究探討將儲存於 2% 福馬林液內，於 4°C 下保存 14 個月之犬蛔蟲受孕卵感
染小鼠第一天至第四百六十九天後，藉顯微鏡檢視與病理學的結果評估孵化的幼蟲對
小鼠的眼部損傷情形。每次犧牲三隻感染鼠與二隻同齡未感染鼠，藉解剖顯微鏡檢視
左眼是否出現幼蟲，右眼部分則加以包埋後進行組織病理學檢測。結果顯示本實驗之
42 隻不同時間的感染鼠之左眼均無檢測到有幼蟲，然而，右眼部分則分別於第 56、
140 和 168 天感染組的其中 1 隻感染鼠被觀察到出現眼部病理變化。幼蟲入侵眼
部所造成的主要病變是視網膜損傷、虹膜睫狀體炎、視神經乳頭炎和淺層梗塞。於 3
組感染鼠中，位於視網膜內的幼蟲並無被發炎細胞浸潤的情形。本報告為首篇關於自
長期培養的犬蛔蟲卵孵育的幼蟲可引起鼠眼部犬蛔蟲症的研究。本結果進一步擴展了
對於犬蛔蟲受孕卵致病性的知識。

關鍵詞：犬蛔蟲，ICR 小鼠，受孕卵，眼球犬蛔蟲症

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