

Blood–brain barrier impairment with enhanced SP, NK-1R, GFAP and Claudin-5 expressions in experimental cerebral toxocariasis

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SUMMARY

Infection by Toxocara canis in humans may cause cerebral toxocariasis (CT). Appreciable numbers of T. canis larvae cross the blood–brain barrier (BBB) to invade the brain thus causing CT. In the present studies, we evaluated the BBB permeability and BBB injury as assessed by the cerebral Evans blue (EB) concentration as well as by pathological changes and glial fibrillary acidic protein (GFAP) expression in T. canis-infected mice monitored from 3 days (dpi) to 8 weeks post-infection (wpi). The vasodilation neuropeptides, the expressions of substance P (SP) and its preferred binding neurokinin-1 receptor (NK-1R) as well as claudin-5 of tight-junction proteins associated with BBB impairment were also assessed by Western blotting and reverse-transcriptase polymerase chain reaction. Results revealed that BBB permeability increased as evidenced by a significantly elevated EB concentration in brains of infected mice. BBB injury appeared due to enhanced GFAP protein and mRNA expressions from 4 to 8 wpi. Leukocytes might have been unrelated to BBB impairment because there was no inflammatory cell infiltration despite T. canis larvae having invaded the brain; whereas markedly elevated SP protein and NK-1R mRNA expressions concomitant with enhanced claudin-5 expression seemed to be associated with persistent BBB impairment in this experimental CT model.

Keywords blood–brain barrier, cerebral toxocariasis, claudin-5, glial fibrillary acidic protein, NK-1 receptor, substance P

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INTRODUCTION

Toxocara canis is an intestinal nematode of canines, the eggs of which are unembryonated when passed in the faeces of dogs and cats into the environment. Under optimal temperature and humidity conditions, the eggs develop into embryonated eggs, and when they are swallowed by paratenic hosts, such as rodents or humans, *T. canis* larvae hatch in the small intestine and gain access to other organs via the portal venous circulation. Humans acquire *T. canis* infection by accident ingestion of embryonated eggs contaminating soil, water, or foods, which may lead to ocular toxocariasis or visceral larva migrans (1).

Cerebral toxocariasis (CT) is predominantly caused by *T. canis* larval invasion of the brain. Although most human CT cases do not present significant clinical neurological signs due to the light parasitic burden (2), some CT cases with severe neurological disorders such as eosinophilic meningitis, encephalitis, myelitis, arachnoiditis and epilepsy have been reported (3–5).

The blood–brain barrier (BBB), formed by highly specialized brain endothelial cells and astrocytes which are enclosed by a vascular basement membrane (6), is a selective barrier that protects the central nervous system (CNS) microenvironment from the systemic circulation by limiting the transport of immune cells and molecules into the brain parenchyma (7). The barrier properties of neural blood vessels are attributed mainly to the presence of complex tight junction (TJ) networks among endothelial cells (8). Accumulated evidence has revealed that claudins, one component of tight junction proteins (TJPs), are key molecules in TJ assembly (9). Claudins expressed in endothelial cells of neural tissues include claudin-1, -3, -5 and -12, and they are thought to be candidate molecules responsible for endothelial barrier function (10). A study of claudin-5-deficient mice disclosed that claudin-5 is indispensable for the barrier function against small molecules by neural blood vessels (11).

Glial fibrillary acidic protein (GFAP), a member of the intermediate filament family, provides support and strength

to astrocytes, and GFAP's function may assist in maintaining the protective barrier that allows only certain substances to pass between blood vessels and the brain. In the presence of BBB injury, astrocytes may react by rapidly producing more GFAP (astrogliosis) in response to the injury (12), thus GFAP expression has been used to evaluate the status of BBB injury in CNS infections and disorders (13–15).

Some microbes and parasites invading the CNS might cause meningitis or encephalomeningitis which is frequently accompanied by the occurrence of BBB impairment (13,16,17). It is thought that leukocyte transmigration from the peripheral blood to the brain plays an important contributing role to BBB impairment; thus, the presence of leukocytes in the brain is one important index of BBB impairment due to CNS infections (13,17).

The neuroactive peptide, substance P (SP) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) produced by nerves, endothelial cells, and cells of the immune system, belongs to the tachykinin family of neuropeptides and is the most abundant neurokinin in the CNS. It elicits its effects by stimulating the neurokinin-1 receptor (NK-1R), which is highly expressed in the CNS and preferred by SP (12). Enhanced SP and NK-1R expressions are reported to be highly associated with BBB impairment in HIV infection (18,19).

In the present studies, we evaluated the BBB permeability and BBB injury as assessed by cerebral Evans blue (EB) concentrations as well as by pathological changes and GFAP expression in *T. canis*-infected mice monitored from 3 days (dpi) to 8 weeks post-infection (wpi). The vasodilation neuropeptides, SP and its preferred binding receptor, NK-1R, as well as caludin-5 expressions associated with the BBB impairment were also assessed by Western blot analysis and reverse-transcriptase polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Parasites and the experiment protocol

Toxocara canis eggs were obtained from adult female worms, and embryonated eggs were prepared according to our previous study (20). Female ICR mice aged 6–8 weeks were obtained from the Centre for Experimental Animals, Academia Sinica, Taipei, Taiwan. Mice were housed in the animal facility of Taipei Medical University and maintained on commercial pellet food and water *ad libitum*. The viability of *T. canis* embryonated eggs was assessed by a light-stimulation method before use (20). Each mouse was infected with about 250 embryonated eggs in 100 µL of water by oral intubation.

Infected mice were divided into two groups. One group was used to assess the status of the BBB permeability (BP group), and the other group was used to evaluate the status

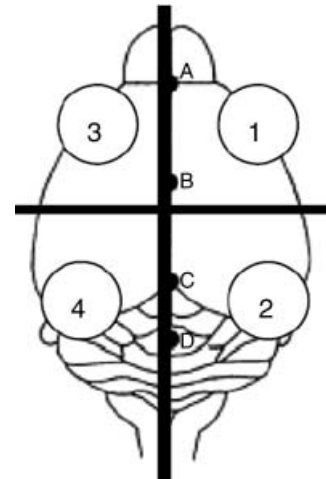


Figure 1 Relative parts of mouse brains used for the various assays in this study (a). The first part was for a pathological study (b), the second part was for a larval recovery study (c), the third part was for Western blotting, and (d) the fourth part was for the RT-PCR analysis.

of BBB injury (BI group). Both groups of infected mice were deeply anaesthetized with ether and killed by heart puncture at 3 dpi, and 1, 4 and 8 wpi. On each date, three infected mice and two age-matched uninfected mice were sacrificed.

In the BP group, the ventricles of the brain of each mouse were removed after EB injection into the tail vein, then the brain was ground with 1.0 mL PBS; after centrifugation, the supernatant was measured and the sediments were further processed for larval recovery studies. In the BI group, after the skull of the mice was opened, the brain was divided into four parts for various studies. The first part was processed for pathological studies, the second part was subjected to acid-pepsin digestion for a larval recovery assay, while the third and fourth parts were processed for Western blotting and RT-PCR assessments, respectively (Figure 1). All animal experiments were carried out in accordance with institutional Policies and Guidelines for the Care and Use of Laboratory Animals, Taipei Medical University and all efforts were made to minimize animal suffering.

BBB permeability assessment in the BP group of infected mice

A volume of 200 µL of 2% (w/v) solution of EB in phosphate-buffered saline (PBS) was injected into the tail vein of a mouse (21). One hour later, the ventricles of the brain of a mouse were removed after it was anaesthetized with ether and killed, and they were ground with 1.0 mL PBS in a glass-tissue grinder with a Teflon pestle. The extract was then centrifuged at 10 000 *g* (Hettich Universal Zentrifugen 16R, Tuttlingen, Germany) for 10 min at room temperature.

The optical density (OD) of the supernatant was read at a 595-nm wavelength in a colourimeter (Molecular Devices Vmax, Kelowna, CA). The sediments were further processed for larval recovery studies.

***Toxocara canis* larval invasion into the brain assessed by a larval recovery study in the BP and BI groups of infected mice**

Larvae in the brain were recovered by a method described in our previous study (22). Briefly, the brain specimen from each infected mouse was individually ground in a Waring blender (Tatung, Taipei, Taiwan). Each sample was digested in 50 mL of a pepsin/HCl solution (pH 1–2, 10 000 IU, Sigma, Steinheim, Germany) for 3 h at 37°C. After the addition of water, the digest was centrifuged (at 250 *g* for 10 min), and the larvae in the sediment were counted in a Petri dish at 100× magnifications under an inverted microscope (Olympus, Tokyo, Japan).

BBB injury assessed by pathological changes and immunohistochemical detection of GFAP expressions in the BI group of infected mice

A brain specimen from each mouse was separately fixed in 10% neutral buffered formalin for at least 24 h and embedded in paraffin for pathological studies and immunohistochemical detection of GFAP. Several serial sections cut at 5-µm thicknesses of each brain specimen from each infected and uninfected mouse were processed using standard procedures and stained with haematoxylin-eosin (H&E) for the pathological study.

For the immunohistochemical studies, brain sections from infected and uninfected mice were deparaffinized and rehydrated using descending ethanol gradients before further processing. Immunohistochemical detection of GFAP was performed as described by Balasingam *et al.* (23). Briefly, endogenous peroxidase activity was blocked by 3% hydrogen peroxide (Merck, Taufkirchen, Germany). An avidin/biotin blocking kit (SP2001, Vector, Burlingame, CA) was used to block the endogenous avidin/biotin activity to reduce background staining. To eliminate nonspecific staining, Fc receptors were blocked with diluted normal goat serum (X0907, Dako, Carpinteria, CA) for 30 min at room temperature in a humid chamber. Sections were then incubated for at least 12 h at 4°C with rabbit antimouse GFAP polyclonal antibodies (pAbs) (cat. no. RB-087, Neomarkers, Fremont, CA) diluted in PBS at 1 : 160. Sections were then washed with 0.05% Tween 20–Tris–HCl buffer three times for 5 min each. Immunohistochemical detection kits (K4003, Dako) were used to detect GFAP-expressing cells by incubating them with horseradish peroxidase-conjugated goat anti-rabbit antibodies for 40 min at room temperature. The

presence of peroxidase was detected using chromogen 3,3-diaminobenzidine (DAB) (K3468, Dako). Sections were counterstained with Gill's haematoxylin (H3401, Vector), dehydrated, and mounted in mounting medium (H5000, Neomarkers). In order to confirm the results of the staining, a normal mouse brain section was used as a positive control. Specificity was ascertained by treating positive control sections as above but omitting the primary antibodies. In addition, for negative controls, we used normal mouse sections subjected to normal rabbit serum as the primary antibody.

BBB injury assessed by Western blotting detection of SP, GFAP and claudin-5 protein expressions in brains of the BI group of infected mice

The method for the Western blot analysis was as described by Ueberham *et al.* (24), with modifications. Briefly, brain specimens from the BI group of infected and uninfected mice were homogenized and lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma, Saint Louis, MO) on ice for at least 1 h. After precipitation, proteins were harvested by centrifugation at 10 000 *g* and 4°C for 10 min, then stored in liquid nitrogen for further studies. Before loading in the gel, protein extracts were boiled for 5 min. In total, 50 µg proteins per lane was electrophoresed in a 6–18% SDS/PAGE mini gel; after which proteins were electrically transferred onto a membrane (Hybond-P polyvinylidene fluoride transfer membrane) for 2 h. Membranes were blocked in 10% PBS/skimmed milk, and then mouse anti-SP monoclonal antibodies (mAbs) (catalogue No. ab14184, Abcam, Cambridge, UK) or rabbit anti-GFAP (catalogue No. RB-087, Neomarkers) or claudin-5 pAbs (catalogue No. sc-28670, Santa Cruz Biotechnology, Santa Cruz, CA) were added for 2 h at 37°C. After several washes, a peroxidase-conjugated rabbit antimouse or goat antirabbit IgG secondary antibody (1 : 10 000), was added to the membranes and incubated at 37°C for 30 min. A Western Lightning® kit (Perkin-Elmer Life Sciences, Boston, MA) was used to detect the immunoreactions. The optical density of the immunoreactive bands was detected with a Typhoon 9000D cabinet equipped with ImageQuant software (GE Life Sciences, Fairfield, CT). The relative multiples of the targeted protein were expressed as an optical density relative to that of the uninfected control group of mice.

BBB injury assessed by RT-PCR detection of NK-1R, GFAP, and claudin-5 messenger (m)RNA expressions in brains of the BI group of infected mice

Total RNA was isolated from murine brains using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) following the manufacturer's protocol. RT-PCR was performed

using a JumpStart™ RED HT RT-PCR Kit (Sigma). Briefly, 1 µg of total RNA was reverse-transcribed using oligo (dT)₂₃ as the primer and enhanced avian myeloblastosis virus reverse transcriptase (eAMV-RT) (Sigma) in a 20-µl reaction mixture. The resulting complementary (c)DNA was appropriately diluted, and the diluted cDNA was amplified using JumpStart REDTaq® DNA polymerase with the following primers: GFAP sense, 5'-GAA TGG CCA CTA AGG CAG TC-3' and antisense, 5'-TGC ACT CCC TCT CTC CTG TT-3' (25); NK-1R sense, 5'-TTC CCC AAC ACC TCC ACC AA-3' and antisense, 5'-AGC CAG GAC CCA GAT GAC AA-3' (26); and claudin-5 sense, 5'-GAC TGC CTT CCT GGA CCA C and antisense, 5'-TGA CCG GGA AGC TGA ACT C (8). Amplified products were electrophoresed on 1.5% agarose gels containing ethidium bromide. The resulting gel bands were visualized in an UV-transilluminator, and the optical density of bands was determined with a Typhoon 9000D series cabinet (GE Life Sciences) equipped with ImageQuant software (GE Life Sciences). The relative amount of the targeted gene was expressed as the optical density relative to that of GAPDH. Three independent cDNA preparations were analysed in each experiment.

Statistical analysis

All data from the larval recovery, immunohistochemical staining, Western blot, and RT-PCR analyses are reported as the mean ± SD. Two-way analysis of variance (ANOVA) followed by one-way ANOVA was used for data analysis. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Numbers of *T. canis* larvae recovered from brains of the BI group but not the BP group of infected mice increased with time

In the BP group, no larvae were recovered from the brains at 3 dpi or 1 wpi. The highest mean (± SD) number of recovered *T. canis* larvae was found at 4 wpi (5.8 ± 3.9), but had declined at 8 wpi (2.5 ± 1.2) (Figure 2). In the BI group, the numbers of larvae recovered greatly increased from 1 (0.3 ± 0.6) to 8 wpi (5.0 ± 7.0) (Figure 2). All of the recovered larvae from both groups were viable as examined by their active motility.

BBB permeability increased with time in the BP group of infected mice

Overall, the OD value of EB extracted from brains of the BP group of infected mice measured at 595 nm increased with

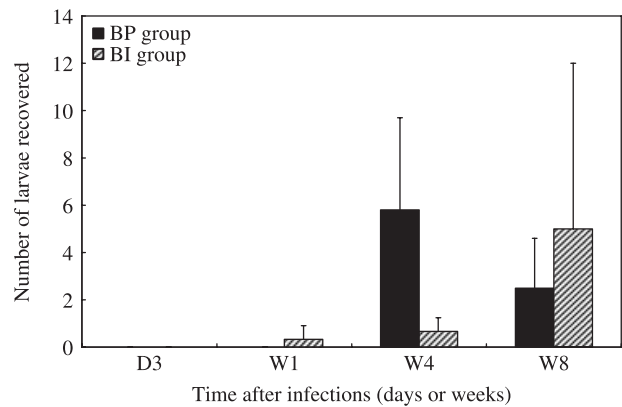


Figure 2 Recovered numbers of larvae from brains of the BP and BI groups of *Toxocara canis*-infected mice. Larval numbers are given as the mean ± SD of larvae harvested from brains of mice infected with *Toxocara canis* embryonated eggs, from 3 dpi to 8 wpi.

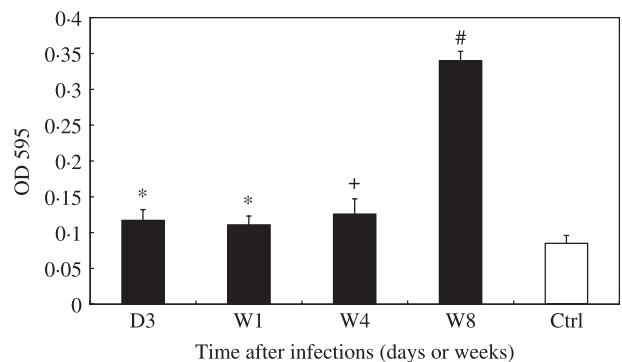


Figure 3 Changes in the amount of Evans blue in the brains of *Toxocara canis*-infected mice. Three to eight mice were in each group. The amount of Evans blue expressed as the optical density measured at 595 nm was significantly higher in infected than uninfected mice (# $P < 0.001$; + $P < 0.01$; * $P < 0.05$).

time in the trial. It was observed to have increased at 3 dpi (0.12 ± 0.02 , $P < 0.05$) and it remained high from 1 (0.11 ± 0.01 , $P < 0.05$) to 4 wpi (0.132 ± 0.02 , $P < 0.01$) compared to that in uninfected mice (0.09 ± 0.01); whereas, the value drastically climbed to 0.34 ± 0.01 ($P < 0.001$) at 8 wpi (Figure 3).

Toxocara canis larvae invaded the area around the choroid plexus without eliciting inflammatory cell infiltration, yet BBB injury was present as evidenced by enhanced GFAP expression in the BI group of infected mice

Pathologically, no inflammatory cell infiltration was found in the experimental groups of infected mice at 3 dpi or 1 wpi

(data not shown). Although larvae were found in or close to the choroid plexus without noteworthy inflammation or injury to the cerebral parenchyma of infected mice at 4 (data not shown) and 8 wpi (Figure 4a) as seen in H&E-stained sections, the immunohistochemical assessment, however, showed numerous astrocytes with significant GFAP expression in the cerebral parenchyma near the choroid plexus in infected mice at 4 (data not shown) and 8 wpi (Figure 4b). In contrast, insignificant GFAP expression was detected in the age-matched control group of uninfected mice at 4 (data not shown) and 8 wpi (Figure 4c).

SP, GFAP and claudin-5 protein expressions as well as NK-1R, GFAP and claudin-5 mRNA expressions were enhanced in brains of the BI group of infected mice

When *T. canis* larvae invaded the brains, there were no changes in protein levels of SP or GFAP during 1 wpi, however, both levels significantly increased as seen at 4 and 8 wpi, with 8.9- and 149.8-fold higher levels for SP and 26.4- and 57.0-fold higher levels for GFAP, respectively (Figure 5a,b). In contrast, claudin-5 expression was observed to increase early from 3 dpi onwards in the trial, and its expression was markedly elevated at 1 wpi by 2.9-fold; however, it reached the highest levels at 8 wpi at 5.5-fold (Figure 5a,b). The RT-PCR revealed that NK-1R and GFAP mRNA expressions were largely concomitant with the protein expressions, but both exhibited significantly elevated expressions at 4 wpi, thereafter they nearly ceased or had declined at 8 wpi (Figure 6a,b); whereas, claudin-5 mRNA expression coincided with the protein expression in the trial (Figure 6a,b).

DISCUSSION

Although most of the effects of brain involvement in human CT are likely to be cryptic and not easily observed or explained (1), *T. canis* larvae sometimes cause CNS disorders, for example, partial epilepsy (5,27,28), cerebellar ataxia (2), and eosinophilic meningoencephalitis (3). BBB impairment was surmised to be related to CNS disorders in CT since it is well known that meningitis and meningoencephalitis caused by many viral, bacterial, fungal, and even parasitic agents might cause BBB impairment (13,29–32), but until very recently information about parasitic helminth infections causing BBB impairment has been very limited (13).

Because CT in humans and mice shares similar pathogenesis and migratory pathways (1), it might be possible to detect CT-associated BBB injury in a murine model. Particularly in mice inoculated with a low dose of *T. canis* eggs, the low burden of *T. canis* larvae (an average larval burden of 6 larvae/brain; range, 0–15 larvae/brain) appearing in the brain and altered changes in murine behaviour are likely to

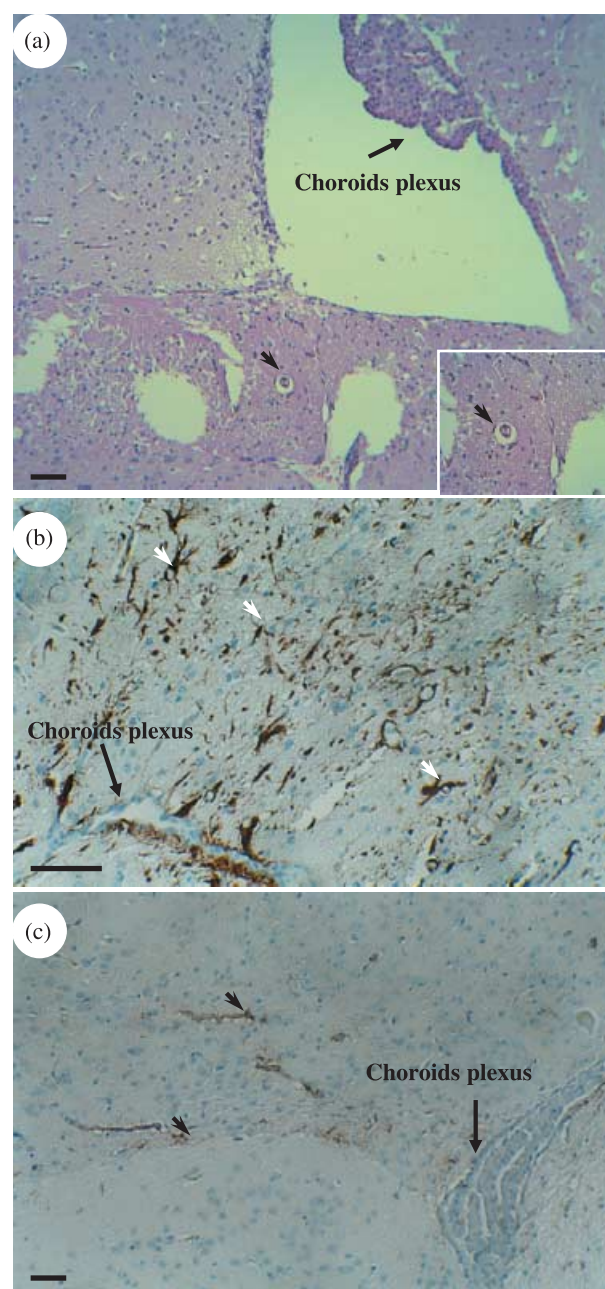


Figure 4 Representative pathological changes and glial fibrillary acidic protein (GFAP) expression in brains of *Toxocara canis*-infected mice by H&E and immunohistochemical staining. Larvae (arrowhead) present in or near the choroid plexus (arrow) showing that there was no inflammatory cell infiltration in the brains of infected mice at 8 wpi (a). Tissue sections were stained with haematoxylin-eosin. Bar = 50 μ m. Astrogliosis with apparent GFAP expression (arrowhead) was observed in the parenchyma near the choroid plexus (arrow) in brains of infected mice at 8 wpi (b). In contrast, insignificant GFAP expression (arrowhead) near the choroid plexus (arrow) was detected in brains of age-matched uninfected mice at 8 wpi (c). Bar = 50 μ m.

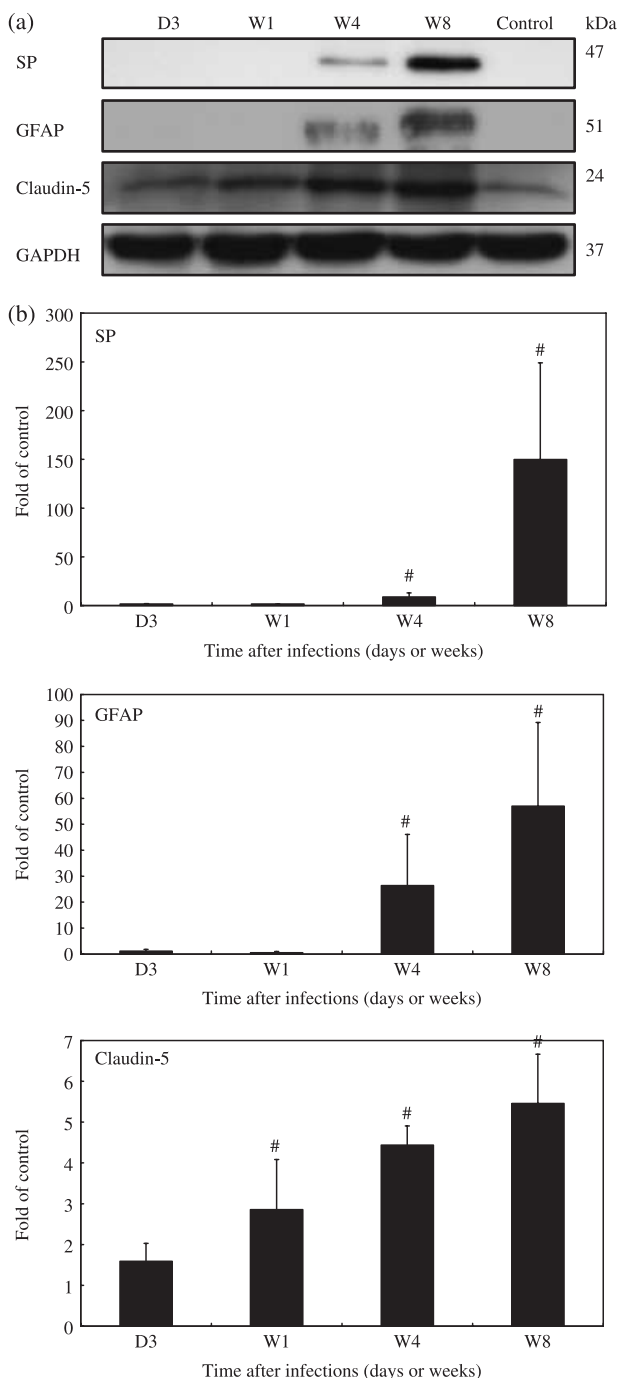


Figure 5 Expression analyses of substance P (SP), glial fibrillary acidic protein (GFAP), and claudin-5 protein in brains of *Toxocara canis*-infected mice. (a) Protein levels of SP and GFAP in brains of infected mice from 3 dpi to 8 wpi as assessed by Western blotting. (b) Relative multiples were generated as described in Fig. 5b. The error bars indicate the standard deviation (SD), and the superscript indicates a significant difference from the control; $^*P < 0.01$, $^{\#}P < 0.001$.

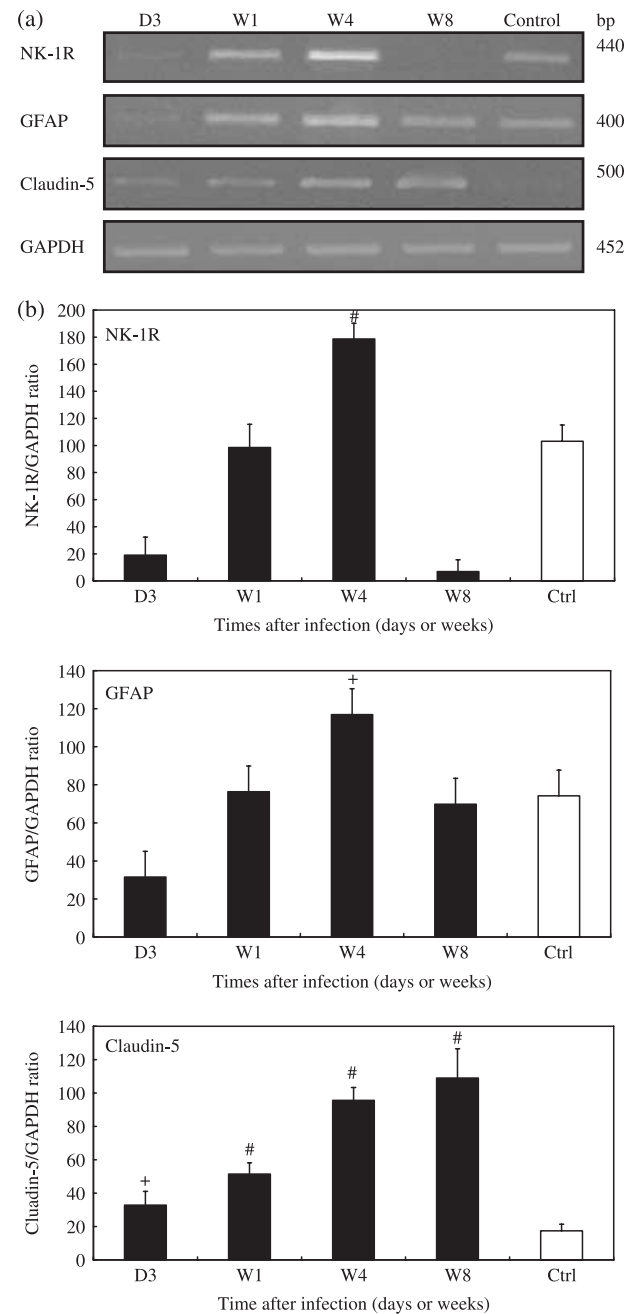


Figure 6 Expression analyses of neurokinin-1 receptor (NK-1R), glial fibrillary acidic protein (GFAP), and claudin-5 messenger (m)RNA in brains of *Toxocara canis*-infected mice. (a) Expression levels of NK-1R, GFAP, and claudin-5 mRNA in brains of infected mice from 3 dpi to 8 wpi were assessed by RT-PCR. (b) The relative amounts of NK-1R, GFAP, and claudin-5 mRNA were calculated based on the optical density relative to that of GAPDH. The error bars indicate the SD, and the superscripts indicate a significant difference from the control; $^*P < 0.01$, $^{\#}P < 0.001$. Three to eight mice per group were examined.

more realistically reflect the situation in humans and wild rodents with toxocariasis (33). The present results of mean larval burdens per brain of 4.2 ± 2.3 and 2.9 ± 3.0 larvae/brain in the BP and BI groups, respectively, are close to that reported by Cox and Holland (33). In addition, Dubinsky *et al.* (34) examined brains of 476 small mammals from Slovakia and found the numbers of larvae to range from 1 to 13 per brain, with the peak average being 4.2 ± 4.1 larvae/brain in animals collected from a suburban location. The low numbers of larvae harboured by the brains in BP and BI groups of infected mice described in this paper are also very similar to those described by Dubinsky and colleagues.

How microbial pathogens are able to cross the BBB and cause CNS infections remains incompletely understood. It was proposed that microbial pathogens might cross the BBB transcellularly, paracellularly, and/or by means of infected phagocytes (14), and alteration of the BBB permeability was observed in CNS infections by various microbial pathogens (35–37). Several techniques are used to monitor BBB permeability. The observation of EB in the brain 1 h after injection into the tail vein is the most convenient method (21). However, it is difficult to distinguish the degrees of BBB permeability by subjective judgement of the colour of a brain stained with Evans blue. The measurement of quantities of EB in the brain was achieved in this study by extracting this dye from the brain and then measuring it with a colourimeter. Thus, the kinetics of BBB permeability changes in mice could be obtained after infection with *T. canis*.

The present results showed that the BBB permeability increased with the infection duration and that an abnormal BBB permeability did not seem to be related to the numbers of *T. canis* larvae invading the brains as seen in the BP group, although increased numbers of larvae with time were seen in the BI group. This discrepancy in the trend of larval recovery might be explained by an increased trend in larval recovery found in the BI group possibly being ascribed to the portions of the brain examined were predominantly the cerebrum and cerebellum; in contrast, the whole brain was used to detect larvae thus resulting in fluctuant changes in larval recovery found in the BP group. Several studies, based upon a single dose of *T. canis* eggs, reported larvae in a nonrandom distribution within the brain (38,39). Summers *et al.* (40) recorded larvae from the cerebrum including heavily myelinated tracts of the corpus callosum and internal and external fornix capsules, as well as the cerebellum including the cerebellar peduncles and cerebellar medulla in Binghamton Heterogeneous stock mice. Good *et al.* (39) also found that the majority of larvae were recovered from the telencephalon (cerebral cortex) and cerebellum; yet larvae were undetected in the mesencephalon (midbrain) and only rarely found in the medulla, diencephalon, and olfactory bulb in outbred CD1 mice. However, Kayes and

Oakes (41) previously indicated that fluctuations in larval numbers might reflect the ability of larvae to migrate into and out of the sampled tissues including the brain over time.

Although pathological findings showed that *T. canis* larvae could invade the area surrounding the choroid plexus, they did not elicit inflammatory cell infiltrations or cerebral parenchymal injury in the brains as examined by several H&E-stained brain sections from the BI group, which is very similar to previous findings reported by other researchers (20,40,42). Since it is widely accepted that the presence of leukocytes in the brain is an important feature of the presence of BBB impairment as seen in meningitis caused by various microbial pathogens including parasites (13,29,43), it seems to be a little difficult to explain BBB impairment caused by leukocyte transmigration due to a lack of evidence of leukocyte infiltration in the brains of *T. canis*-infected mice as assessed by pathological changes with H&E staining. Why no inflammatory cell infiltration was detected in brains in experimental CT might be partially explained by *T. canis* larvae *per se* being able to mimic a host's antigenic components, therefore avoiding attracting inflammatory cell infiltration, which provides larvae a way to escape a host's immune attacks, or the brain *per se* having some mechanism to diminish inflammation in order to protect it from severe injuries caused by inflammatory storms (1). Nevertheless, BBB injury indeed occurred as evidenced by enhanced GFAP expression (astrogliosis) in the brains of infected mice. Several studies have indicated that astrocytes might react by rapidly producing more GFAP in response to BBB injury, and marked GFAP expression was proposed as being a way to help maintain the BBB integrity damaged by invading microbial pathogens (12). Noteworthy, we also observed that BBB disruption was present early in the infection and prior to the appearance of larvae in the brains as seen at 3 dpi, suggesting that BBB disruption might facilitate entry of *T. canis* larvae into the brain; thereafter, BBB impairment became more evident possibly as a consequence of the migration of larvae into the brain and their subsequent residence there. The postulation is supported by that enhanced claudin-5 expression of TJ proteins was concomitantly present with elevated BBB permeability early from 3 dpi onward in this trial, indicating the presence of damage to TJ integrity, which resulted in disruption of the barrier properties of neural vascular endothelial cells in early infection. Until recently, three distinct types of integral membrane proteins are known to be localized at TJ: occludin (44), junctional adhesion molecule (45), and claudin (46). However, several studies including gene knockout analyses revealed that TJ strands can be formed without occludin (47). junctional adhesion molecule with a single transmembrane domain was recently shown to associate laterally with TJ strands, but not to constitute the strands *per se* (48). In

contrast, claudin is now believed to be a major constituent of TJ strands (49) and claudin-5 was found specifically in endothelial cells, in large amounts especially in the brain endothelial cells, leading to the idea that claudin-5 may be directly involved in the establishment of the BBB (50). Although substantial literature exists showing that BBB permeability in acute brain injury is associated with reduced claudin-5 levels, several studies have indicated that inflammatory pain may increase both BBB permeability and claudin-5 expression (51). However, disruption of barrier function has been reported with both decreased and increased claudin-5 expression, indicating complex regulation of the protein within the TJ. Brooks *et al.* (51) indicated that disruption of paracellular permeability to [14 C] sucrose correlated with both a decrease and a sixfold increase in claudin-5 expression at 24 and 72 h post-Complete Freund's Adjuvant (CFA)-inducing pain, respectively. Reduced expression of claudin-5 has been associated with increased permeability in several previous studies (52,53). While transfection of claudin-5 into epithelial cells with a low electrical resistance enhances barrier function (54), over-expression of claudin-5 results in a loss of barrier function and increased permeability and paracellular volume of distribution (55). The BBB integrity was compromised as has been observed with many infections, for example, bacteria such as *Vibrio cholera* can cause alterations in TJ proteins which in turn cause openings in the BBB and subsequent CNS infection (56), while viruses such as HIV-1 can stimulate rat brain endothelium cells to secrete SP to increase the permeability (57). However, each of these diseases is supposed to involve the dysregulation of TJs that maintain the BBB.

Since larvae invaded into the brains without eliciting noteworthy inflammatory cells infiltration in the brains; inflammatory cells did not play a role in disruption of the BBB integrity in this experimental CT model, other factors such as cytokines or neuropeptides are proposed as being involved in BBB impairment. Since substantial evidences have indicated that SP plays an important role in inflammatory pain that may result in increased BBB permeability through activation of glial cells by releasing a variety of substances, for example, proinflammatory cytokines thus increasing the excitability of nearby neurones (12), we may propose that SP-inducing pain may involve in persistent BBB impairment as well as enhanced claudin-5 expression in experimental CT due to the findings that SP protein expressions as well as NK-1R mRNA expressions were enhanced in the brain. However, further experiments using SP/NK-1R antagonist should be carried out to exam the hypothesis.

Recently, Hamilton *et al.* (58) indicated that expression of potent vasodilation enzyme, inducible nitric oxide synthase (iNOS) is enhanced in the brains of inbred strains of *T.*

canis-infected mice (BALB/c and NIH) and this response is detected as early as 3 dpi and persists up to 97 dpi. Since SP has been confirmed to be capable of augmenting nitric oxide production and iNOS gene expression in murine macrophages (59), whether SP has a role in promoting the iNOS expression in neural vascular endothelial cells which affects TJ integrity thus leading to BBB disruption warrants further investigation.

It is difficult to explain why NK-1R and GFAP mRNA expressions in infected mice are weaker than those in uninfected mice in early infection at 3 dpi, since at that time no larvae invade the brains. We may, however, propose that in early infection some unclear mechanisms, for example, cytokines or stress may affect the NK-1R and GFAP mRNA stability to attenuate their expressions in the brains of infected mice; however, this postulation and their corresponding pathophysiological function in early infection should be further tested. Of particular interest, it was indicated that SP and its preferred receptor (NK-1R) have important roles in the induction of epilepsy by eliciting lasting BBB disruption (60). Since much evidence has indicated a high association between partial epilepsy and *T. canis* infection (5,27,28), further experimental work should focus on assessing whether it will be helpful using an SP or NK-1R antagonist to treat or prevent BBB impairment and or epilepsy in CT.

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CONFLICTS OF INTEREST STATEMENT

The authors have no conflicts of interest concerning the work reported in this paper.

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