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Disruption of the blood–brain barrier in pigs naturally infected with *Taenia solium*, untreated and after anthelmintic treatment

Cristina Guerra-Giraldez^{a,b}, Miguel Marzal^a, Carla Cangalaya^a, Diana Balboa^a, Miguel Ángel Orrego^a, Adriana Paredes^a, Eloy Gonzales-Gustavson^c, Gianfranco Arroyo^c, Hector H. García^{a,b,d}, Armando E. González^c, Siddhartha Mahanty^{a,e}, Theodore E. Nash^{a,e,*}, and The cysticercosis working group in Peru

Cristina Guerra-Giraldez: cristina.guerra@upch.pe; Miguel Marzal: miguel.marzal.m@upch.pe; Carla Cangalaya: carla.cangalaya.l@upch.pe; Diana Balboa: dsbalboa@gmail.com; Miguel Ángel Orrego: miguel.orrego.s@upch.pe; Adriana Paredes: adriana.paredes.a@upch.pe; Eloy Gonzales-Gustavson: gonzaleseloy@yahoo.com; Gianfranco Arroyo: arroyogianfranco@gmail.com; Hector H. García: hgarcia@jhsph.edu; Armando E. González: agonzale@jhsph.edu; Siddhartha Mahanty: smahanty@niaid.nih.gov

^aLaboratorio de Inmunopatología en Neurocisticercosis, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru

^bDepartamento de Ciencias Celulares y Moleculares, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru

^cFacultad de Medicina Veterinaria, Universidad Nacional Mayor de San Marcos, Lima, Peru

^dUnidad de Cisticercosis, Instituto Nacional de Ciencias Neurológicas, Lima, Peru

^eLaboratory of Parasitic Diseases, NIAID, NIH, Bethesda, MD, USA

Abstract

Neurocysticercosis is a widely prevalent disease in the tropics that causes seizures and a variety of neurological symptoms in most of the world. Experimental models are limited and do not allow assessment of the degree of inflammation around brain cysts. The vital dye Evans Blue (EB) was injected into 11 pigs naturally infected with *Taenia solium* cysts to visually identify the extent of disruption of the blood brain barrier. A total of 369 cysts were recovered from the 11 brains and classified according to the staining of their capsules as blue or unstained. The proportion of cysts with blue capsules was significantly higher in brains from pigs that had received anthelmintic treatment 48 and 120 h before the EB infusion, indicating a greater compromise of the blood brain barrier due to treatment. The model could be useful for understanding the pathology of treatment-induced inflammation in neurocysticercosis.

Keywords

Blood–brain barrier; Neurocysticercosis; *Taenia solium*; Praziquantel

*Corresponding author. Address: 9000 Rockville Pike, Bldg 4/Rm 424, Bethesda, MD 20892, USA., tnash@niaid.nih.gov (T.E. Nash).

1. Introduction

Neurocysticercosis (NCC) results from the infection of the brain with cysts (larvae) of the cestode tapeworm *Taenia solium*. Pigs, the natural intermediate host, may become heavily infected and harbor thousands of cysts, mostly in the muscles but also in the brain. Humans serve as the sole definitive hosts when they ingest raw or undercooked pork containing cysts that develop into adult tapeworms. These grow in the small intestine and produce a chain of increasing mature proglottids containing thousands of ova, which are shed in the feces (García et al., 2005). By accidentally ingesting cysts and ova via the fecal-oral route, humans can also serve as intermediate hosts and may be infected and develop disease when cysts invade the brain. Disease presentations are very variable and depend on the number and location of cysts and degree of associated inflammation (García et al., 2003; Nash and Garcia, 2011; Nash et al., 2006). Viable cysts incite little inflammation, but their degeneration provokes an inflammatory response, which is directly or indirectly the cause of much of the symptomatology. Although anthelmintic treatment kills cysts, the inflammation this elicits is deleterious and significantly complicates the therapy and management of the infection.

The pathophysiology of treatment-induced injury in NCC and how best to suppress accompanying inflammation have not been well studied in multicystic cysticercosis. A major impediment to detailed pathophysiological studies is the lack of a usable model of NCC. Although *T. solium* infected pigs have been employed before to study the course of infection (Sikasunge et al., 2009), and efficacy of treatment (Gonzalez et al., 2012; Sikasunge et al., 2008), there have been few studies investigating the pathogenesis of post-treatment inflammation.

Degenerating and dead cysts are surrounded by inflammatory cells that infiltrate from the periphery into the central nervous system (Álvarez and Teale, 2006; Rickard and Williams, 1982). This is accompanied by disruption of the blood brain barrier (BBB; reviewed by Abbott et al. (2010), Hawkins and Egleton (2008), Moody (2006), Nico and Ribatti (2012), Rubin and Staddon (1999), Wolburg et al. (2009)), characterized by increased vascular permeability. The basis of BBB disruption is likely multifactorial, but there is strong evidence in transgenic mice pointing to a failure of the terminal region of the astrocytic endfeet to attach to endothelial cells of blood vessels and/or to produce the modulating factors that form and maintain the BBB through various signaling events (Araya et al., 2008; Janzer and Raff, 1987; McCarty, 2005). The vital dye Evans blue (EB) (Evans and Schulemann, 1914) forms a stable complex with serum albumin (Allen and Orahovats, 1950; Patterson et al., 1992) and is used even today to assess BBB disruption (Kaya and Anishali, 2011; Manaenko et al., 2011; Okamura et al., 2010; Zhang et al., 2013). After EB injection, a brain with an intact BBB will remain unstained, since albumin is prevented from diffusing into the interstitium. However, if vascular permeability increases, the dye attached to albumin diffuses into the subcellular interstitial tissues, identifying areas of BBB disruption that accompanies pathological processes like inflammation (Clasen et al., 1970; Hawkins and Egleton, 2008; Menkin and Menkin, 1930; Vaz et al., 1998).

We present a model for studies of the BBB in NCC, based on EB extravasation in animal NCC and the effects of anthelmintic drugs on the brain.

2. Methods

2.1. Animals

Eleven naturally infected pigs were obtained from Huancayo, an endemic zone in the central Peruvian Andes. Infection status was determined locally by the presence of cysts in the tongue and later confirmed by immunoblotting. Three pigs remained untreated (U) and the rest received a single dose of praziquantel (100 mg/kg; Saniquantel 10%, Montana SA, Peru) administered orally 2 or 5 days before EB staining (T48 and T120, respectively). All procedures were approved by the Animal Ethics and Well being Committee (CEBA) of the Facultad de Medicina Veterinaria, Universidad Nacional Mayor de San Marcos.

2.2. Staining with EB

After anesthesia with intramuscular ketamine (10 mg/kg; Ket-A-100, Agrovét Market SA, Peru) and xylazine (2 mg/kg; Dormi-Xyl 2, Agrovét Market SA, Peru), the carotid vein of the pig was cannulated, and EB (2% in 0.85% NaCl; Sigma–Aldrich, St. Louis, MO) was delivered intravenously at a total dose of 80 mg/kg together with sodium pentobarbital (25 mg/kg, Halatal, Montana SA, Peru). The pigs were maintained under sodium pentobarbital sedation (20–25 mg/kg every 45 min or as needed up to a maximum of 120 mg/kg). After 2 h, the animal was euthanized with a lethal dose of pentobarbital and perfused with chilled saline solution or 10% formalin (3.7% formaldehyde in PBS, pH 7.2), using a peristaltic pump for 20 min. A longer exposure to the dye was rejected to reduce the stress on the animal of prolonged sedation or two consecutive surgical procedures. Lower exposures complicated the organization of the activities when operating on more than one pig. For logistical reasons, 1–3 animals were studied in each experiment and data from six experiments were pooled.

2.3. Collection of samples

Immediately after perfusion, the brain was removed, placed on dry ice and cut into 10-mm slices ice to expose parenchymal cysts; photographs documented the findings. Cysts and their capsules (identified by a layer of collagen fibers with or without inflammatory infiltrates) and surrounding brain tissue were placed in saline solution. Selected samples were fixed with formalin (infused after perfusion) or stored in RNALater® (QiaGen, Hilden, Germany) for histological and molecular assays that are described elsewhere (unpublished data, CWGP). Cysts were also collected from skeletal muscle; these were removed without the surrounding tissue and placed in saline. Brain cysts with blue (EB stained) or clear capsules were counted (enumerated).

3. Results

3.1. Macroscopic observations

On gross inspection of whole and sliced brains, EB extravasation allowed identification of cysts with BBB breakdown; blue capsules were found in all infected pigs. Brains from pigs

that had received the anthelmintic drug, praziquantel, showed more abundant blue cysts and the staining was noticeably darker than in untreated pigs, suggesting a high degree of leakage (Fig. 1).

3.2. Frequencies of cysts/capsules with EB extravasation

A total of 369 cysts were collected from all 11 infected pigs; parasitic loads were very variable, with as few as 1 and up to 95 cysts per brain with an average of 33.5 ± 28.1 (SD) cysts per brain. The staining or lack of it was evident in the capsules around the cysts: 239 were blue and 130 clear (unstained), but the distribution in the U and T groups differed statistically ($p < 0.0001$ by Fisher's exact test), as can be seen in Table 1 and Fig. 1. The increase in the proportion of blue stained cysts was more apparent in pigs that received EB 48 h after anthelmintic treatment (blue/clear ratios between 4.5 and 8.0) than in those stained 120 h after treatment (blue/clear ratios between 1.0 and 5.0), compared with pigs not given antiparasitic treatment (blue/clear ratios between 0.4 and 7.0).

4. Discussion

The well-established methodology of injecting EB to observe disruption of the BBB was used in naturally infected untreated pigs as well as pigs previously treated with praziquantel. Uninfected control pigs were not included, since they would not have cysts for comparisons. It is also well documented that the complex formed by EB and albumin is excluded from the brain parenchyma by a fully functional BBB, so brain tissue from uninfected controls would have remained unstained by EB (Okamura et al., 2010; Marchi et al., 2010). This has been a consistent observation in a number of studies in which EB was used to evaluate the integrity of the BBB in experimental settings, such as *Plasmodium* infections in mice (Egima et al., 2007) and ultrasound-induced vascular disruption in rats (Wei et al., 2013). In the case of NCC, involvement of the BBB has been reported in the murine model by demonstrating migration of leukocytes in the brain of mice experimentally infected with *Mesocestoides corti* (Alvarez and Teale, 2006). Histopathological studies of brains from naturally infected pigs also demonstrated alterations in the vasculature and cellular infiltrates suggestive of the BBB disruption, but these studies did not show direct evidence of leakage from the vasculature (Sikasunge et al., 2009). By directly showing vascular disruption around cysts, the present model should allow the study and characterization of the cerebral inflammation induced by anthelmintic treatment in this disease.

Interestingly, 77% of cysts of all eight treated pigs (201/261) had blue stained capsules compared to only 35% of cysts (38/108) in the three untreated pigs, a significantly higher proportion and a strong suggestion that treatment with PZQ had resulted in an increase in the permeability of the BBB. As altered permeability of the BBB allows for greater infiltration of inflammatory cells in the affected region, this correlates with the observation that symptoms of infections in the CNS are exacerbated after treatment, the reason for frequent use of corticosteroids to control this effect (Nash and Garcia, 2011). Although the number of pigs is small and the parasite load too variable to allow exact conclusions, the higher blue/clear ratios of pericystic tissue in brains of pigs after 48 h of treatment with praziquantel and the lower ratios after 120 h match the observation of the appearance of pericystic edema

after 24 h of administration of praziquantel and its disappearance one week later, as documented by Jena et al. (1992) using MRI on NCC patients.

One problem of using naturally infected pigs to study the effects of treatment is the lack of information about the inflammatory response and degree of cyst degeneration before the intervention. Extravasation of the albumin-conjugated dye could be seen in infected animals that had not been treated with the drug, which indicates that the EB infusion (80 mg/kg, intracarotid, 48 h before euthanasia) was adequate for the study of BBB dysfunction in this model. The high blue/clear ratio (7.0; seven blue cysts and one clear one) found in one untreated pig could mean that infection and inflammation in this pig was in an advanced stage, something that cannot be predicted before the experiment.

The blue staining was more evident and more frequent in pericystic tissue in the brains of pigs that had received the anthelmintic drug (Fig. 1). Other studies of these tissues show increased inflammation and associated cyst wall damage after only 48 h of anthelmintic treatment (unpublished data, CWGP). These studies indicate that disruption of the BBB and EB staining was associated with increasing inflammation post treatment.

Disruption of the BBB in brain injury is also evaluated by magnetic resonance imaging (MRI) and methods based in radioactively labeled tracers, but these are more expensive and less practical than EB, which is preferred in most recent experimental studies (Manaenko et al, 2011; Zhang et al., 2013). Using EB staining in infected pigs treated with an anthelmintic drug, we have been able to correlate the BBB disruption with functional changes at the histological and molecular level that occur as a result of treatment (unpublished data, CWGP), which serves, as used here, to accelerate the degeneration of the cysts and the resulting inflammatory response. This method also identifies blood vessels in close association with the cysts, by the presence of EB in the perivascular spaces once the BBB has been compromised. These vessels may perhaps serve the parasite as a source of nourishment but also serve as an access pathway for host inflammatory cells. It is tempting to speculate that post-treatment enhancement in MRI of the brain of patients, useful in their follow-up evaluation (Teitelbaum et al, 1989), may represent the same phenomenon of BBB dysfunction and, thus, could be considered equivalent to EB staining in our model. This correlation has been indeed tested and found positive ($r = 0.981$, $P = 0.019$), comparing changes in the ratio of capillary number stained by EB and MRI signal intensity during traumatic brain injury (Lin et al., 2012).

In summary, in vivo Evans Blue infusion allowed us to directly observe BBB “leakage” in pigs naturally infected with brain cysticercosis, and the effect of an anthelmintic drug. It also helps to gather information about immunological events during the course of NCC and its treatment. This model can potentially prove useful for understanding the mechanisms underlying the adverse effects of anthelmintic treatment and serves as a model to find better drugs, methods to reduce treatment related complications or even to design diagnostic methods to locate points of focal disruption of the BBB for early intervention. The system is currently being applied to study the relationship between disruption of the BBB, inflammation and neurological damage.

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HIGHLIGHTS

- Evans blue was injected in pigs with *Taenia solium* cysts in the brain.
- Leakage of dye around some cysts indicated disruption of the blood–brain barrier.
- The dye leaked more in brains of pigs previously treated with praziquantel.
- The model could help to study treatment-induced inflammation in neurocysticercosis.

Fig. 1.

Coronal sections of an untreated (A) and a praziquantel treated (B) brain after an infusion of Evans blue. *T. solium* infected pigs, one untreated and one treated with praziquantel (100 mg/kg), were administered an intravenous infusion of Evans Blue (80 mg/kg; see Methods), and euthanized 2 h later for retrieval of the brain. Clear and dark arrows point to one clear and one blue stained capsule or pericystic area in each brain, respectively.

Number and proportion of Evans Blue stained cysts in infected untreated pigs and pigs treated with praziquantel 48 h or 120 h before dye injection *

Table 1

Group	Pigs (N)	Number of cysts		Proportion blue/clear	Fisher's exact test		
		Total	Blue				
Untreated	3	108	70	38	0.5 (0.4, 1.3, 7.0)	U vs. all T	$P < 0.0001$
Treated, 48 h	4	184	25	159	6.4 (4.5, 5.4, 6.9, 8.0)	U vs. T48	$P < 0.0001$
Treated, 120 h	4	77	35	42	1.2 (1.0, 1.0, 1.1, 5.0)	U vs. T120	$P = 0.0106$
All treated	8	261	60	201	3.4	T48 vs. T120	$P < 0.0001$

* Fisher's exact test.