

Detection of fibronectin and laminin in *Toxoplasma gondii* tissue cysts: immunocytochemical assays

Detecção de fibronectina e laminina em cistos teciduais de Toxoplasma gondii: ensaios imunocitoquímicos

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ABSTRACT

Aims: To analyze the existence and distribution of some matrix proteins in tissue cysts of *Toxoplasma gondii*. **Methods:** Laminin and fibronectin in tissue cysts of *Toxoplasma gondii* were detected by confocal microscopy and transmission electron microscopy. **Results:** Ultrastructural immunocytochemistry showed both glycoproteins in the granular region of tissue cysts, cystic matrix, micronemes, rhoptries, dense granules and rarely at the membrane of bradyzoites of *Toxoplasma gondii*. **Conclusions:** The presence of both laminin and fibronectin in secretory organelles and in the apical region of bradyzoites suggests that exocytosis of these glycoproteins can contribute to their interaction with host cells, besides composing the cyst matrix of *Toxoplasma gondii*.

Keywords: *Toxoplasma gondii*/chemistry; *Toxoplasma gondii*/citology; CYSTS/parasitology; IMMUNOCYTOCHEMISTRY; GLYCOPROTEINS; LAMININ; FIBRONECTINS; MICROSCOPY, CONFOCAL; SCANNING TRANSMISSION ELECTRON MICROSCOPY

INTRODUCTION

The apicomplexan parasite *Toxoplasma gondii* (*T. gondii*) has the ability to infect a wide range of intermediate hosts, including mammals and birds. To survive within infected hosts, *T. gondii* undergoes profound metabolic and morphological changes by differentiating into a cyst. Tissue cysts are resistant to the host immune response and current drug treatments, contributing to the success of *T. gondii* to maintain the chronic phase of the disease.¹⁻⁴

Studies on the characterization of the cyst wall of *T. gondii*, as well as informations about the contribution of the host cell in the formation of this

structure, have been little explored. The cyst wall is important for the maintenance and integrity of the tissue cyst in the host cell for long periods and it appears that it is produced by modifications of the parasitophorous vacuole membrane, during invasion of tachyzoites and their transformation into bradyzoites.^{5,6} Despite the knowledge that the cyst wall limits the presentation of antigens to the host, contributing to its silent intracellular persistence, few is known about the composition of this structure.⁷⁻¹⁰

Extracellular matrix elements constitute a complex network of different types of versatile proteins and polysaccharides that are synthesized locally and organized in association with the cellular surface. The cell-extracellular matrix interactions seem to be dynamic and reciprocal, staining a bi-directional flow of information that regulates fundamental processes, such as growth, differentiation, migration and cellular recognition.¹¹ Besides the well-known role of extracellular matrix proteins such as fibronectin,

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collagen and laminin in the cytoadhesion of different pathogens, glycosaminoglycans have also been implied in the pathogenesis of infectious processes.^{12,13} Laminin and heparan sulfate proteoglycans act as host cell receptors to a great variety of pathogens, including the *T. gondii*.¹⁴⁻¹⁷ Our present aim was to analyze some matrix proteins in tissue cysts of *T. gondii*, by confocal and ultrastructural immunocytochemistry as tools for the detection of the proteins laminin and fibronectin.

METHODS

Parasites

The strain ME-49 of *T. gondii* was used. The parasites were maintained in C57BL/6 mice female, weighing about 12-18 grams, inoculated with about 50 cysts/animal. After 4-8 weeks the mice were sacrificed to obtain brain cysts.

Cysts isolation and purification

The methodology used was adapted and modified from the protocols described by Freyre and Popiel et al.^{18,19} and modified by Guimarães et al. (2008, 2009).^{20, 21}

Transmission electron microscopy

The tissue cysts were fixed for 1 h at 4°C with 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2). After fixation, the cysts were washed in PBS and post-fixed for 30 min with 1% OsO₄ in 0.1 M cacodylate buffer plus 5 mM CaCl₂ and 3.5% sucrose at room temperature. The cells were then washed in the same buffer, dehydrated in acetone and embedded in PolyBed 812 resin. Thin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss EM 10C transmission electron microscope.

Fluorescence microscopy

Recently isolated cysts were fixed for 5 min at room temperature with 4% paraformaldehyde (PFA) diluted in PBS, washed 3 times for 10 min with PBS, incubated for 1 h at 37°C in a blocking solution (4% bovine serum albumin, 5% goat serum, 0.01% saponin in PBS), and then incubated overnight at 4°C with rabbit anti-laminin (Sigma Chemical Co. code L9393) or anti-fibronectin (Sigma Chemical Co. code F3642) antibodies diluted 1:500 in PBS. After incubation with the first antibody, the cysts were washed 3 times for 10 min in PBS containing 4% BSA, and then

incubated for 1 h at 37°C with an anti-rabbit antibody labeled with tetramethylrhodamine (TRITC, Sigma Chemical Co. code T 6778) diluted 1:300 in PBS. Thereafter, the cysts were washed 3 times for 10 min in PBS, incubated for 10 min with 0.1 µg/mL DAPI (4',6-Diamidino-2-phenylindole, Sigma Chemical Co. code D9542) diluted in PBS, washed once in PBS, and then mounted on slides with 2.5% DABCO (1,4 Diazabicyclo [2.2.2] octane – Triethylenediamine – antifading, Sigma Chemical Co. code D2522) in PBS containing 50% glycerol. Controls were performed by omission of the primary antibody. The samples were examined with a LSM 410 confocal laser scanning microscope (Zeiss, Germany) with a 543 nm emission filter Laser and a LP 570 nm excitation filter.

Ultrastructural immunocytochemistry

After chemical fixation (4% PFA, 0.1% glutaraldehyde, 0.2% picric acid in 0.1M sodium cacodylate buffer, pH 7.2, 1 h at 4°C) the tissue cysts were washed with the same buffer and incubated during 30 min at 4°C in PBS containing 50 mM ammonium chloride to block free aldehydes. The cells were dehydrated in methanol (30%, 50% 70%, 90%) at -10°C and then embedded in metanol: Lowicryl (2:1, 1:1 1:2) for 24 h and pure Lowicryl during 5-7 days at -20°C. For immunolabeling, ultra-thin sections were picked up in nickel grids, incubated in Tris buffered saline (TBS) containing 50 mM ammonium chloride, washed in TBS containing 1% BSA and 1% Tween, and subsequently incubated overnight at room temperature with rabbit anti-laminin [Sigma Chemical Co. code L9393] or anti-fibronectin [Sigma Chemical Co. code F3642] antibodies diluted 1:10 in TBS + 1% BSA + 1% Tween. After this incubation, the sections were washed and finally incubated for 1 h at room temperature with protein A conjugated to 10 nm colloidal gold particles (Sigma Chemical Co. P1039) diluted 1:10 in TBS + 1% BSA + 1% Tween. The sections were contrasted with uranyl and lead citrate and then examined in a Zeiss EM 10C transmission electron microscope. Controls were performed by omission of the primary antibody.

RESULTS

The fibronectin expression in *T. gondii* tissue cysts was analyzed by confocal laser scanning microscopy. The results demonstrated a heterogeneous expression of fibronectin in these cysts (Figure 1 a-f). Considering that the cysts were permeabilized with saponin, the labeling was observed apparently in the cystic wall,

extending to the interior of the cyst, being possible to observe in a same sample cysts with an intense and uniform labeling (Figure 1 a-c), with punctual distribution (Figure 1 d-f) or with discrete labeling (data not shown). The use of ultrastructural immunolabeling allowed the localization of fibronectin at the surface or in the area close to the membrane of the cyst wall (granular region) and also in vesicles located in that region. It was not possible to detect fibronectin labeling in the cyst membrane, although differences in the fibronectin expression were also detected among the cysts, as observed by immunofluorescence. Besides the labeling at the granular region, it was also observed labeling on the cystic matrix (Figure 2a, b), as well as inside the bradyzoites, specifically in the micronemes, dense granules, rhoptries and rarely in the plasma membrane (Figure 2a, b). Some parasites presented labeling in the apical region (Figure 2b). The

control assays made in the absence of the first antibody presented no labeling, confirming the specificity of the reaction (data not shown).

The presence of laminin in tissue cysts, evaluated by confocal laser scanning microscopy, revealed a heterogeneous expression (data not shown). By ultrastructural analysis, a low expression of laminin was observed in the surface of the cysts, while an expressive labeling was found in the granular region in some cysts and also inside vesicles located in the granular region (Figure 3a, b). Labeling was also observed in the cystic matrix (Figure 3a); inside the bradyzoites, specifically in the rhoptries (Figure 3b); in the amylopectin granules (Figure 3c); and also, as a weak labeling, in the micronemes (Figure 3c). Some parasites presented labeling in the apical region (Figure 3c). The control of the reaction, made with omission of the first antibody, presented no labeling.

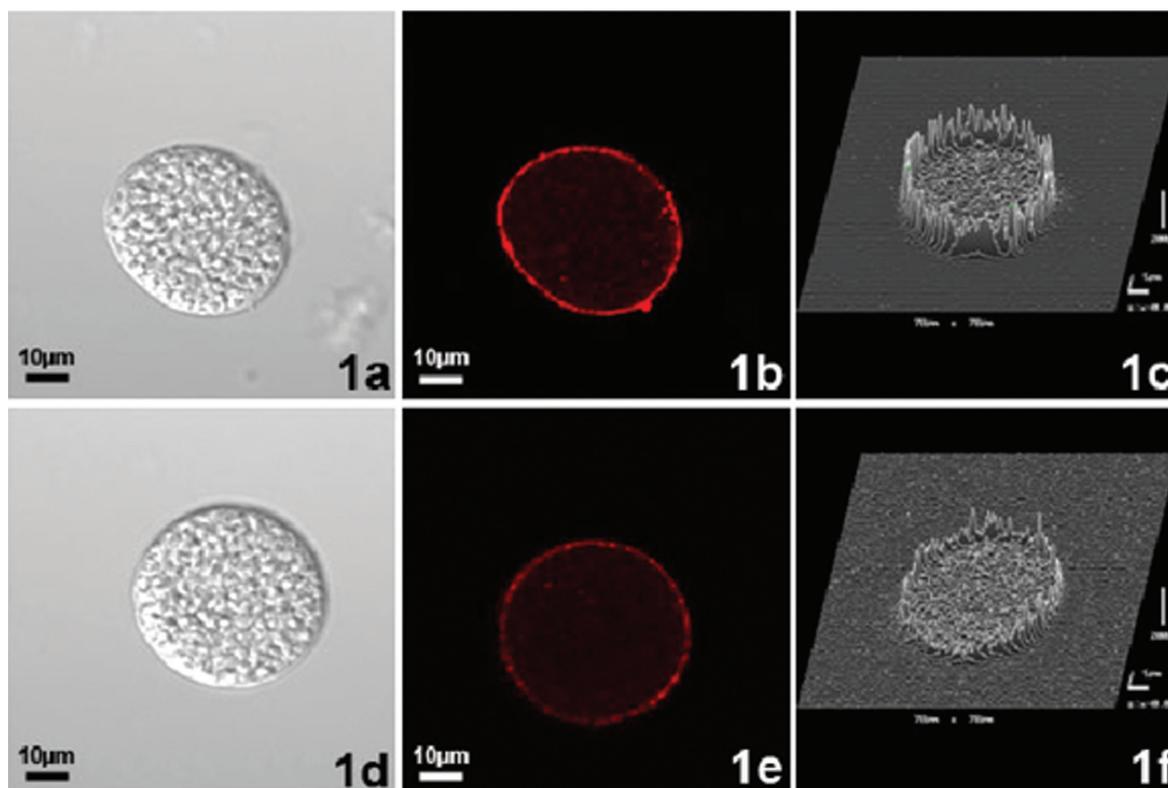


Figure 1. Fibronectin expression in tissue cysts of *Toxoplasma gondii* after immunolabeling and confocal microscopy. **1a, 1d)** Different tissue cysts observed by interference microscopy showing the cystic wall and several parasites in its interior. **1b, 1e)** Optical sections of different *Toxoplasma gondii* cysts revealing heterogeneity in the labeling for fibronectin in the cystic wall with extension to its interior. Some punctual labeling is observed inside the cysts. **1c, 1f)** Histograms of intensity of fluorescence for fibronectin in tissue cysts based on figures 1b and 1c, respectively.

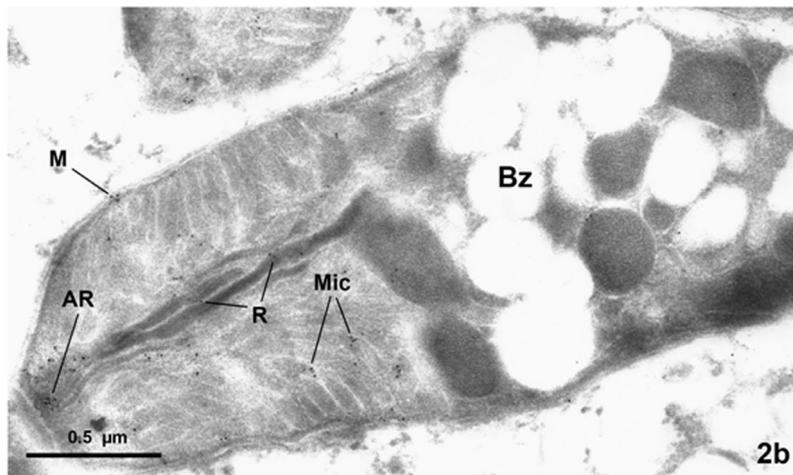
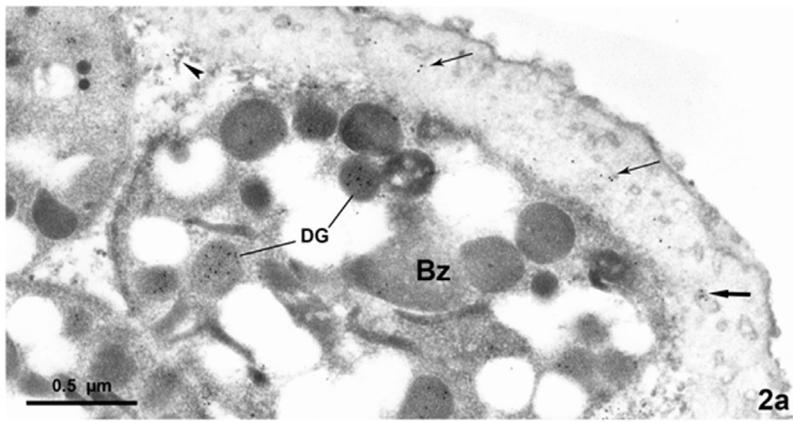


Figure 2. Fibronectin expression in tissue cysts of *Toxoplasma gondii* by ultrastructure immunocytochemistry. **2a, 2b)** Image demonstrates fibronectin in the granular region (arrows) and in the cystic matrix (arrowhead). Colloidal gold particles are evident inside bradyzoites, specifically in the dense granules (DG), rhoptries (R), micronemes (Mic) and apical region (AR). The labeling can also be observed in vesicles in the granular area of the cyst (large arrow). Rare particles of colloidal gold are observed in the bradyzoites (Bz) membrane (M).

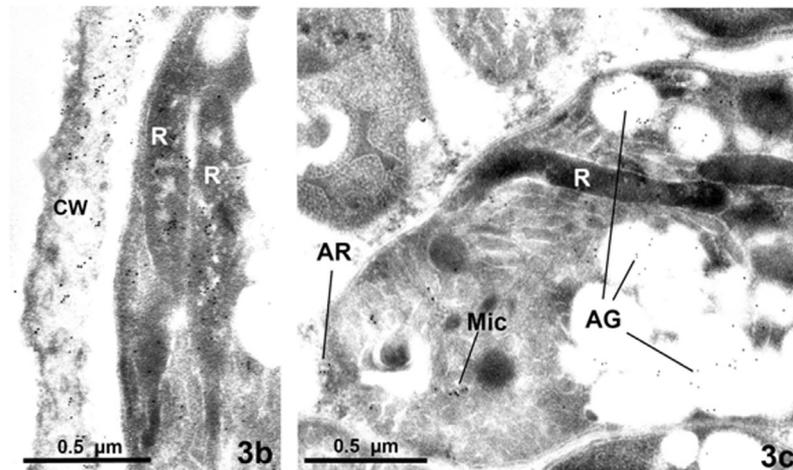
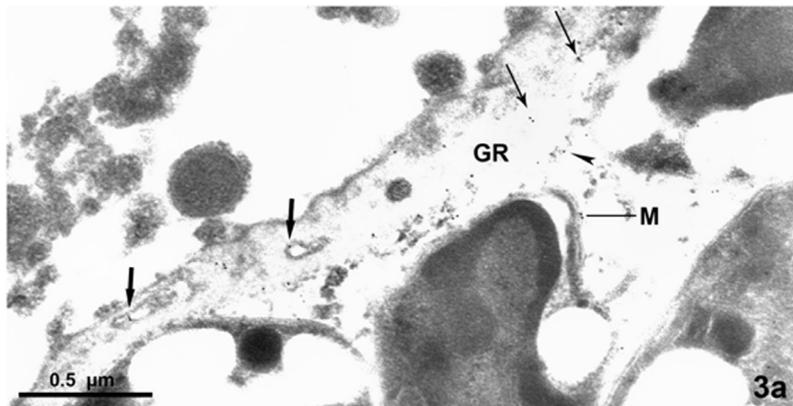


Figure 3. 3a-c) Laminin expression in tissue cysts of *Toxoplasma gondii* by ultrastructure immunocytochemistry. Ultrathin section of a tissue cyst showing colloidal gold particles in the granular region (GR, arrow), in vesicles localized in this region (large arrow), in the matrix (arrowhead) and rare particles in the membrane of the parasite (M). **3b)** Positive reaction for laminin in the rhoptries of bradyzoite (R) and cyst wall (CW). **3c)** Laminin expression in amylopectin granules (AG), rhoptries (R), micronemes (Mic), and apical region (AR) of bradyzoites.

DISCUSSION

Fibronectin and laminin in tissue cysts of *T. gondii* were detected by confocal microscopy, demonstrating a heterogeneous pattern in label intensity. Ultrastructural immunocytochemistry showed both glycoproteins in the granular region of tissue cysts, cystic matrix, micronemes, rhoptries, dense granules and rarely at the bradyzoites membrane. The ultrastructural aspects of tissue cysts of the *T. gondii* ME-49 strain described here are in accordance to the description of other groups.^{5,20,21}

The analysis by confocal microscopy of extracellular matrix proteins expression (fibronectin and laminin) in tissue cysts did not reveal any pattern in the labeling intensity. This should probably happen by variations in size of the cysts inside a same isolate and inside a same populations, and thus it was not possible to correlate the labeling intensity of these glycoproteins with the size of the cysts. If we consider that in the intermediary host there is a infection for long time (assymtomatic form), that allows the production of new tissue cysts with different levels of maturity. It could also be explained the differences in labeling intensity by maturity degree of the tissue cysts isolated from a same brain.^{5,22}

Studies that identify the fibronectin and laminin glycoproteins in *T. gondii* tissue cysts are still lacking. Studies regarding extracellular matrix proteins in *T. gondii* were available, using tachyzoite forms to verify the participation of these elements in the parasite adhesion to the host cells. It has been shown that tachyzoite use laminin, probably originating from the host cell in this process.¹⁴⁻¹⁶ In other cellular models, including other protozoa, it is also described the contribution of glycoproteins to important cellular processes.²³

The data presented here showed the presence of both glycoproteins in secretory organelles of *T. gondii* bradyzoites located inside tissue cysts, in the cystic matrix, in the cystic granular region and occasionally, in the membrane that composes the cyst wall. It was also possible to locate fibronectin and laminin molecules in the apical region of bradyzoites, suggesting that exocytosis of these glycoproteins can occur.

We propose that this event could contribute to the establishment of the cellular recognition process between bradyzoites and the host cells, promoting bridges between these elements and their receptors in the surface of the host cells and the parasites. This hypothesis now is under investigation in our group. These series of evidences support the idea that glycoproteins in *T. gondii*, as described in others cellular models, including other protozoa, can contribute to important cellular processes, such as the composition of the cyst matrix, parasites proliferation, adhesion of the parasites to the target cells, and cellular differentiation.

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