

An Alternative Technique to Reveal Polysaccharides in *Toxoplasma gondii* Tissue Cysts

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Ultrathin sections of tissue cysts isolated from the brain of Toxoplasma gondii infected mice were submitted to two different methodologies derived from the periodic acid - Schiff's reagent (PAS) technique. The use of osmium tetroxide vapor as a developing agent of the aldehyde oxidation to reveal polysaccharides with periodic acid resulted in positive reaction in amylopectin granules in bradyzoites, as well as in the wall and matrix of the cysts, with excellent increment of the ultrastructural morphology. This technique can be used for study of T. gondii-host cell intracellular cycle, the differentiation tachyzoite-bradyzoite, and also for the formation of cysts into the host cells.

Key words: *Toxoplasma gondii* - amylopectin granules - tissue cysts - ultrastructural cytochemistry

Infection with the protozoan parasite *Toxoplasma gondii* is endemic throughout the world. This obligate intracellular parasite is able to persist in its hosts by differentiating from the replicative tachyzoite stage into cysts forming latent bradyzoites. The reversible stage differentiation in *T. gondii* makes it an interesting model system of protozoan differentiation. The energy requirements and biosynthetic substrates of coccidian parasites are mostly dependent on carbohydrate metabolism. The reserve of polysaccharides in *T. gondii* is found in amylopectin granules, which are scarce or absent in tachyzoites, but abundant in bradyzoites (reviewed in Dubey et al. 1998, Speer et al. 1999). Bradyzoites enriched with amylopectin granules are colored in red when stained with the periodic acid - Schiff reagent (PAS) technique (reviewed in Dubey et al. 1998) besides being less susceptible to the destruction by proteolytic enzymes, as compared with the tachyzoites (Jacobs et al. 1960, Dubey 1998). In this report we present the characterization of these amylopectin granules in *T. gondii* tissue cysts by two alternative ultrastructural cytochemistry methodologies.

MATERIALS AND METHODS

Tissue cysts - C57BL/6 mice were infected intraperitoneally with 50-100 cysts of *T. gondii* ME-49 strain, an avirulent cyst-forming strain. Brains from 4-8 weeks

infected mice were collected, washed in phosphate buffered saline (PBS) and homogenized in 30% Dextran (w/v) in Hank's balanced salt solution (HBSS) by 5 passages through an 18-23 gauge needle at a concentration of no more than 1 brain/2.5 ml. This solution was centrifuged at 3000 g for 10 min and the pellet containing the tissue cysts was resuspended in HBSS (Freyre 1995, Popiel et al. 1996).

Transmission electron microscopy - Isolated 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 at room temperature with 1% OsO₄ in 0.1 M cacodylate buffer containing 5 mM CaCl₂ and 3.5% sucrose. Cells were then washed in the same buffer, dehydrated in acetone and embedded in PolyBed 812. Unstained thin sections were processed for cytochemistry.

Ultrastructural cytochemistry - To characterize the polysaccharides expression (amylopectin granules) in tissue cysts, we have used two methodologies. Thin sections were collected on gold grids, oxidized for 20 min with 1% periodic acid, quickly washed twice in distilled water, and finally incubated for 72 h, in humid chamber at room temperature, in 2% thiocarbohydrazide (TCH) diluted at 20% (v/v) in acetic acid. These grids were washed successively in decreasing concentrations of acetic acid (10%, 5%, 3%, and 1%) for 1 min each. The polysaccharide revelation was made by incubating the grids for 30 min in an aqueous solution with 1% silver proteinate.

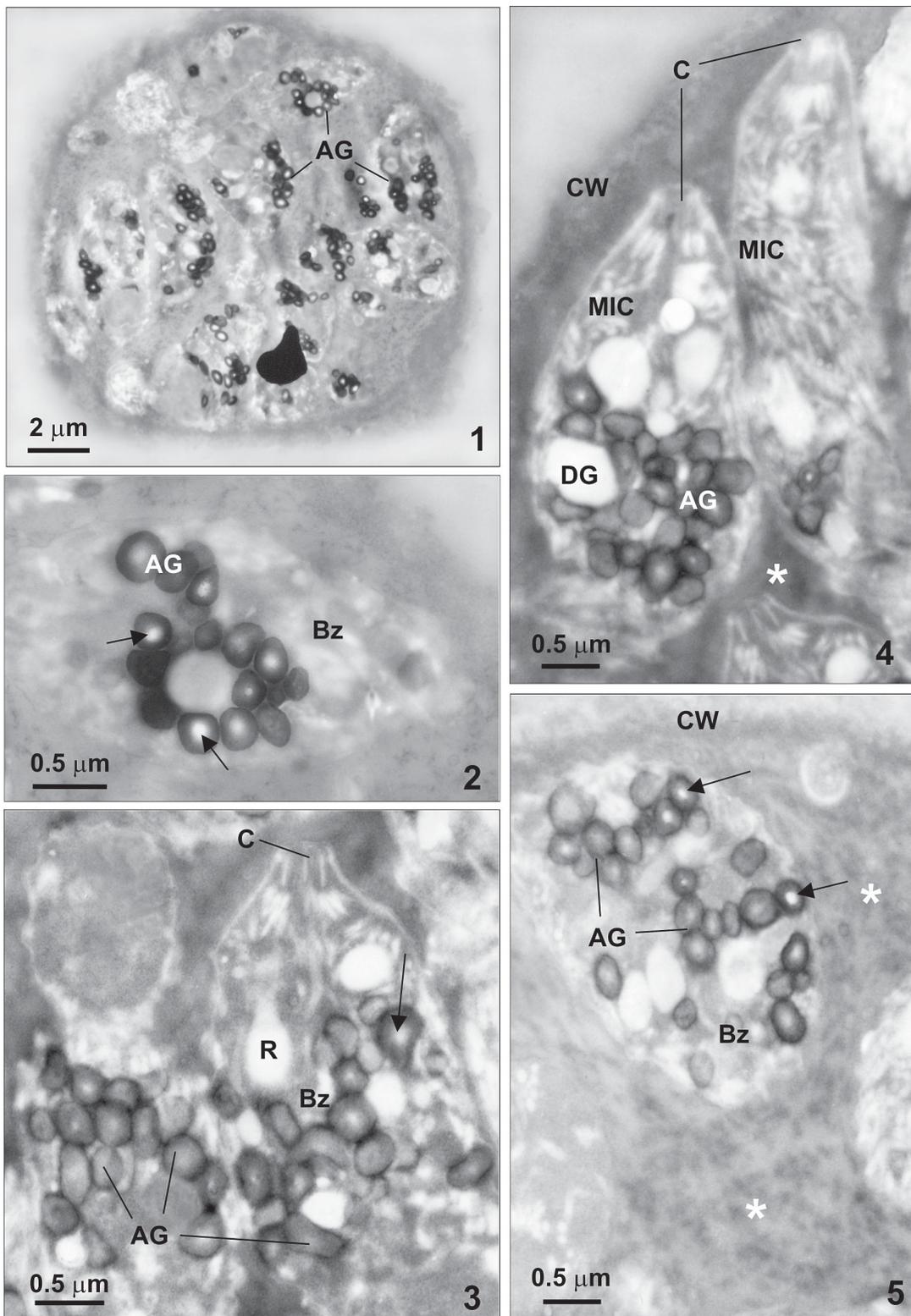
In the second methodology, after the steps described above, the carbohydrate revelation was accomplished by incubation of tissue cysts on grids with osmium tetroxide vapor for 1 min. Using both methodologies after the revelation, these grids were quickly washed once with distilled water, followed by another wash for 10 min. Controls experiments of the reaction were obtained by the omission of the oxidation step by the periodic acid, or the treatment of the cysts with TCH to guarantee that the reaction has been produced by free aldehydes, or by reaction of TCH with the biological material, respectively. Other control was made by omission of the osmium

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Figs 1-2: reaction product for the Thiéry technique employing silver proteinate as developing agent of reaction product. Fig. 1: ultrathin section of a whole cyst containing parasites, the most presenting the electron-dense reaction product located in cytoplasmic organelles, indicative of the presence of amylopectin granules (AG). Fig. 2: detail of the reaction product in AG inside a bradyzoite (Bz). Figs 3-5: modification of the Thiéry technique using the osmium vapor in *Toxoplasma gondii* tissue cysts. Fig. 3: detail showing the AG and the ultrastructural morphology of the parasites (Bz). Some amylopectin granules present the central area lacking the reaction product (arrow); R: rhoptria; C: conoid. Fig. 4: aspects of the reaction product for the osmium vapor showing intense reactivity in AG and in the cystic wall and matrix. A contrast reversion was observed with this technique, allowing a detailed visualization of the structures and organelles of the parasites; CW: cystic wall; MIC: microneme; DG: dense granules; * : matrix. Fig. 5: reaction product observed in AG and in *, appearing as increase in electron-density, indicative of the presence of glycoconjugates.

tetroxide. Finally, the sections were examined unstained in a Zeiss EM 10C transmission electron microscope.

RESULTS

Both methodologies used in the current work revealed polysaccharides in bradyzoites forms of *T. gondii* tissue cysts located in electrondense oval bodies, the amylopectin granules. The granules were observed in large amount mainly in the middle-posterior portion of the parasites (Figs 1-5). Fig. 1 shows the reaction product when silver proteinate was used as a developing agent of the aldehyde reactivity. The reaction product was intense, presenting also a very fine granular deposit in the cyst matrix and in the cyst wall (Figs 1, 2). This reaction was observed in most intracystic parasites, although some parasites presented no reaction product or small intracellular granules (Fig. 1).

When the osmium tetroxide vapor was used as a developing agent for the reaction, besides increasing the electron density of the amylopectin granules inside the cysts, it allowed an excellent visualization of the structures of the parasites, such as internal membranes, conoid, rhoptries and micronemes, which acquired a negative contrast (Figs 3-5). Some granules presented the central area without reaction product (Figs 2, 3, 5). The wall, the granular region, and the matrix of the cysts also showed enhanced reactivity, although with lower intensity (Fig. 5). No reaction product was visualized in cells when TCH, osmium or periodic acid was omitted (data not shown).

DISCUSSION

The alternative method for the detection of *vic diols* in carbohydrates used here, showed to be sensitive and selective, additionally improving the quality of the parasite ultrastructural morphology. The two ultrastructural cytochemistry techniques used in this study to detect polysaccharides and glycoproteins in *T. gondii* tissue cysts using TCH to detect aldehydes produced by the periodic acid are derived from reagent of PAS technique routinely used in light microscopy and adapted for transmission electron microscopy by Hanker et al. (1964) and Thiéry (1967). The Hanker's method used the osmium vapor to reveal the final product of reaction. Later, Thiéry (1967) adapted the technique, facilitating the treatment of ultra-thin sections of specimens embedded in hydrophobic conditions (for example, epoxy resins), obtaining better results in the revelation of the reaction with the use of metallic protein, the silver proteinate. With the introduction of the silver proteinate, the osmium technique was abandoned because it produces less contrast than the silver, which is also less toxic to the operator. A drawback of the silver use is, however, that the silver grains form precipitates that hinder a clean reaction, and thus the reaction needs to be repeated several times, until a clean reaction can be obtained.

Due to the problems with the use of silver, we have

chosen trying to use the osmium technique, and the quality of our results was superior than that obtained with the silver proteinate. The use of osmium allowed to obtain images of high resolution with great contrast of the structural cell components, evidentiating besides the amylopectin granules, the cellular membranes, nucleus, conoid, rhoptries, dense granules and micronemes. This facilitated the structural visualization of the parasites, without the presence of precipitates and without affecting the revelation of the final reaction product.

The method used in this study, can be applied to distinguish between bradyzoite and tachyzoite forms inside the tissue cysts, due to the presence of large amount of amylopectin granules in the bradyzoites, responsible for great polysaccharides accumulation and as material of energy reserve in these parasites. The osmium method showed to be sensitive to delimitate the matrix and cystic wall. Our methodology, besides the above-described advantages, can be used for studies on the interaction between bradyzoites and skeletal muscle cell or other host cells, improving the follow-up of intracellular cycle of *T. gondii*, its reversible differentiation (tachyzoite-bradyzoite), and also during the formation of latent bradyzoites in cysts into the host cells, *in vitro*.

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REFERENCES

- Dubey JP 1998. Re-examination of resistance of *Toxoplasma gondii* tachyzoites and bradyzoites to pepsin and trypsin digestion. *Parasitology* 116: 43-50.
- Dubey JP, Lindsay DS, Speer CA 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 11: 267-299.
- Freyre A 1995. Separation of toxoplasma cysts from brain tissue and liberation of viable bradyzoites. *J Parasitol* 81: 1008-1010.
- Hanker JS, Seaman AR, Weiss LO, Ueno H, Bergman RA, Seligman AM 1964. Osmiophilic reagents: new cytochemical principle for light and electron microscopy. *Science* 146: 1039-1043.
- Jacobs L, Remington JS, Melton ML 1960. The resistance of the encysted form of *Toxoplasma gondii*. *J Parasitol* 46: 11-21.
- Popiel I, Gold MC, Booth KS 1996. Quantification of *Toxoplasma gondii* bradyzoites. *J Parasitol* 82: 330-332.
- Speer CA, Dubey JP, McAllister MM, Blixt JA 1999. Comparative ultrastructure of tachyzoites, bradyzoites, and tissue cysts of *Neospora caninum* and *Toxoplasma gondii*. *Int J Parasitol* 29: 1509-1519.
- Thiéry JP 1967. Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *J Microscopie* 6: 987-1018.