

SHORT COMMUNICATION

Antigen Incorporation on *Cryptosporidium parvum* Oocyst Walls

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Cryptosporidium parvum oocysts are the infective stages responsible for transmission and survival of the organism in the environment. In the present work we show that the oocyst wall, far from being a static structure, is able to incorporate antigens by a mechanism involving vesicle fusion with the wall, and the incorporation of the antigen to the outer oocyst wall. Using immunoelectron microscopy we show that the antigen recognized by a monoclonal antibody used for diagnosis of cryptosporidiosis (Merifluor®, Meridian Diagnostic Inc.) could be found associated with vesicles in the space between the sporozoites and the oocysts wall, and incorporated to the outer oocyst wall by an unknown mechanism.

Key words: *Cryptosporidium* - oocyst wall - antigen

Cryptosporidium parvum is a protozoan parasite belonging to the Phylum Apicomplexa, responsible for diarrhoeic infections in vertebrates. The life cycle completes on the intestinal brush-border, with the excretion of sporulated oocysts with double-layered walls (Current & Reese 1986). As described for other coccidia, the *C. parvum* oocyst wall is supposed to be formed by the serial fusion of two distinct types of granules present at the macrogamete called oocyst-wall forming bodies type I and II (Current & Reese 1986). In the present work we describe the incorporation of molecules to the outer oocyst-wall through a process that apparently does not involve wall-forming bodies, since the oocyst wall is already fully developed.

Oocyst purification - Fecal samples were obtained from spontaneously infected newborn Holstein calves. The samples were diluted in saline solution (0.9% NaCl; w/v) and filtered (1 mm pore) to remove gross material. *C. parvum* oocysts were purified using a cesium chloride gradient, according to Taghi-Kilani and Sekla (1987), without the sucrose preconcentration step. Purified oocysts were stored in saline solution at 4°C until used.

Immunoelectron microscopy - Purified *C. parvum* oocysts were fixed in 2% (v/v) glutaralde-

hyde-formaldehyde in 50 mM cacodylate buffer (pH 7.4) for 2 h at 4°C. The sample was then washed with the same buffer, dehydrated in ethanol and embedded in Spurr (Sigma) for 12 h. After polymerisation for 16 h at 60°C, ultrathin sections were obtained using a diamond knife, and collected on formvar coated nickel grids. The sections were floated on 1% (w/v) bovine serum albumin in PBS (PBSA) for 4 h at 4°C, and incubated with mAb OW3 (primary antibody reagent, murine anti-cryptosporidial mAb, Merifluor™-*Cryptosporidium* kit, Meridian Diagnostic Inc., Sterling & Arrowood 1986) diluted 1:5 in PBSA, for 1 h at room temperature. After washing in PBSA, they were incubated with goat anti-murine IgM serum conjugated with 10 nm gold beads (Sigma) (diluted 1:100 in PBS, added with 0.1% BSA, 0.05% Tween 20 and 5% foetal bovine serum) for 1 h at room temperature. Dry grids were stained with 3% uranyl acetate in water before observed with a Zeiss EM10C electron microscope. Control sections were incubated with an unrelated mouse serum and a monoclonal antibody (mouse monoclonal IgM against rat IgG1, Sigma).

The monoclonal antibody recognised an oocyst-wall antigen >200 kDa (Arrowood & Sterling 1989), and has been widely used for diagnosis of cryptosporidiosis in fecal samples by immunofluorescence detection. Immunodetection using gold-labelled antibodies on ultrathin sections of *C. parvum* oocysts revealed antigen localisation on the oocyst outer surface, displaying an irregular pattern (diffuse agglomerations with different

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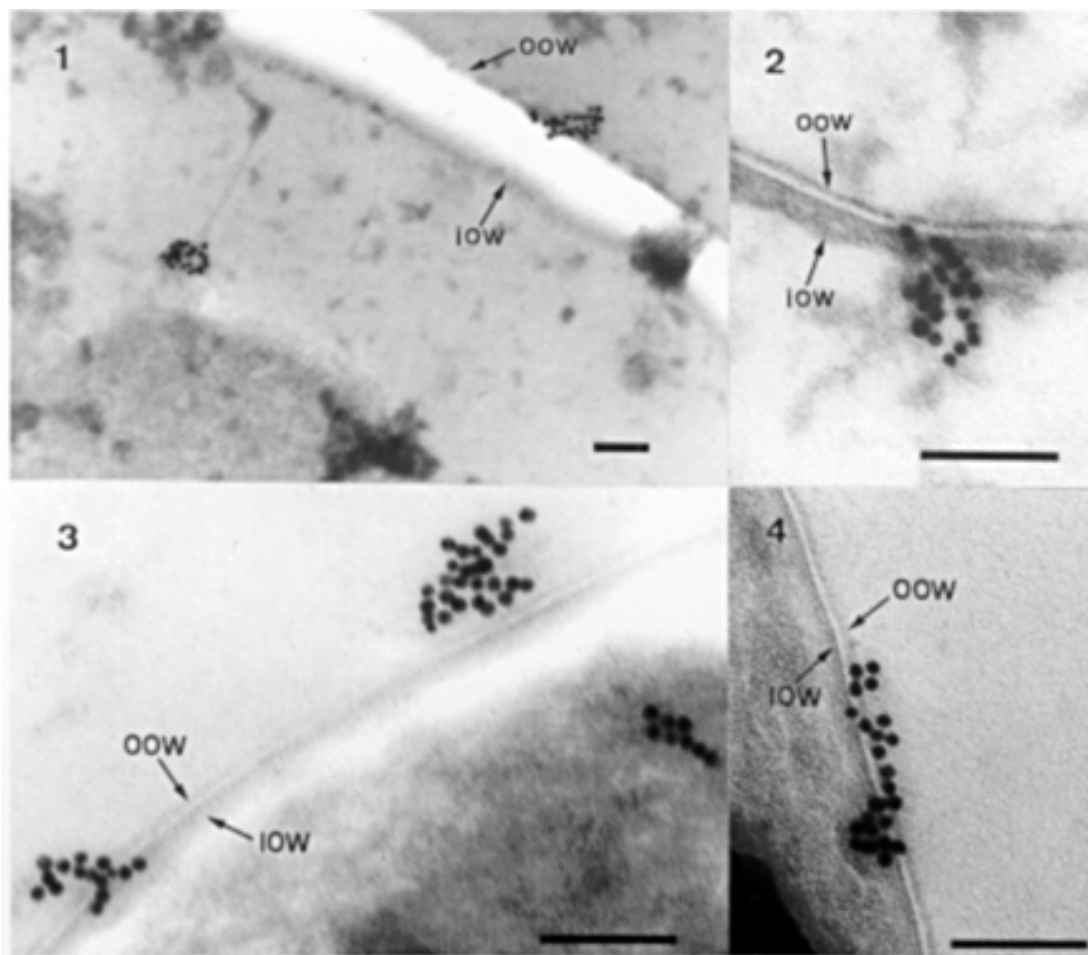
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shapes, Figs 1, 3) with different degrees of attachment to the outer oocyst wall. No labelling was detected when control serum and control monoclonal antibody were used. Surprisingly, the antigen could also be found inside the oocyst, in vacuoles associated with the fibrillous material found between the inner oocyst wall and the sporozoites (Fig. 1), and sporadically between sporozoites and the residual body (data not shown). These vesicles displayed a more regular spherical or ellipsoidal shape, with a mean diameter of 55.1 ± 21.2 nm, while the diffuse agglomerations (irregular blebs) outside displayed 97.9 ± 9.8 nm wide by 59.0 ± 6.4 nm high. In Figs 3 and 4 we could see areas showing labelling at the inner and outer oocyst walls, strongly suggesting that these vesicles were able to release the antigen to the outer oocyst wall, after the oocyst wall is fully developed. This hypothesis is supported by the fact that the antigen could be detected in supernatant obtained by centrifugation (4,000 rpm, 15 min, 4°C) of CsCl puri-

fied oocysts (1×10^9 oocysts) stored in saline solution for two weeks at 4°C. The supernatant was filtered through 0.22 μ m filters, desalted by dialysis in distilled water (using Sephadex G-25, HiTrap™ Desalting columns, Pharmacia), and concentrated by freeze-drying. Dot ELISA revealed the presence of the antigen in this supernatant, showing the labile nature of antigen's attachment to the outer oocyst wall (data not shown).

Reduker et al. (1985) first described the association of knob-like structures, similar to the vesicles shown in the present work, with the inner oocyst wall. The structures shown in that paper, using scanning electron microscopy, displayed similar size and shape like those hereby described. Bonin et al. (1991) also described the presence of electron lucent vacuoles harbouring an antigen recognised by mAb OW-IGO which was detected both inside and outside the oocyst wall, with an unknown nature and function. They suggested a non-wall forming body origin for these vesicles,



Figs 1-4: immuno-electron micrographs showing labeling with mAb OW3 on *Cryptosporidium parvum* oocyst walls. Bars = 0.1 μ m

since the antigen was also found in all sexual forms of the parasite (including microgametes), and in the parasitophorous vacuole, suggesting a secretory nature rather than a wall-forming function. The present work is in agreement with this hypothesis, confirming the possibility for some oocyst wall components to be incorporated after the oocyst wall is fully developed. In a previous work (Entrala et al. 1995) we showed that a significant percentage of recently excreted *C. parvum* oocysts did not show acid-fast staining property, and acid-fast staining property developed with storage time. It is possible that the "immature" oocyst walls may require some components be incorporated in order to display acid-fast staining.

The nature and function of this incorporation of antigens to the outer oocyst wall requires further investigation. The fact that the antigen was easily released from the outer surface of the oocysts may indicate that antigens are continuously incorporated into the oocyst wall, in order to maintain its physiological functions. The data presented here could be important to the understanding of the dynamic nature of the oocyst wall, as well as its importance in *Cryptosporidium* life cycle, such as excystation, autoinfection and resistance to chemical disinfection.

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