The release of secretory vesicle in encysting *Giardia lamblia*

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Abstract

*Giardia* is an intestinal parasite that undergoes adaptation for survival outside the host. It secretes an extracellular cyst wall using a poorly understood process. An encystation-specific secretory vesicle (ESV) was previously described containing cyst wall proteins. The process of release of these vesicles has been suggested to occur after fragmentation of large ESV in small secretory vesicles, followed by exocytosis, but it was not demonstrated. The release of the ESV was studied by transmission electron microscopy. It was observed: (1) the moment of vesicle release; (2) that a large vesicle is exocytosed and does not fragment into small vesicles; (3) membrane fusion is distinct from traditional exocytosis since it is incomplete; (4) the occurrence of membrane fragmentation and that those membranes reseal to form ghosts; (5) these membrane ghosts may be endocytosed, adhered to flagellar surface or/and form empty vesicles in the extracellular medium.

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1. Introduction

*Giardia lamblia* is a protozoan parasite of worldwide distribution. *Giardia* presents the trophozoite form, which colonize the small intestine. In the gut lumen, trophozoites differentiate into cysts, which pass out with the host’s feces. The filamentous cyst wall renders cysts resistant to harsh environmental conditions, and hence, enables the spread of the disease.

Despite its medical and biological importance the process by which trophozoites differentiate into cysts is not fully understood. Of particular interest is the question of how the extracellular wall is deposited by the encysting trophozoite. The cyst wall presents interconnected filaments of 7–20 nm in diameter that contains peptide and carbohydrate moieties, in which 57% is protein and 43% carbohydrate, complexed in an unknown manner [1,2]. An important question concerns the way by which these components are formed and released to the cell surface during the encystation process.

Modification of the culture medium has permitted the completion of the *Giardia* life cycle in vitro [3]. Since the development of the encystation medium, studies on the *Giardia* biology progressed. Reiner et al. [4] described the encystation-specific vesicles (ESV) observed during the process of encystation. According to these authors, the ESV transport cyst proteins to the cell surface for the formation of the cyst wall. Several explanations have been proposed to clarify the mechanisms involved in vesicle release and cyst wall formation [5–7]. Reports have indicated that cyst wall components are released by exocytosis. However, this process has never been documented, and thus the way in which the release occurs is still undefined. With respect to ESVs, it is imperative to determine whether these large vesicles actually secrete their contents directly by fusing with plasma membrane, as has been suggested previously [8]. In addition, as *Giardia* does not present an evident Golgi
complex. Marti and Hehl [9] proposed the occurrence of a complete dispersal of ESV into small secretory vesicles before secretion of cyst wall material, suggesting that the ESV could correspond to Golgi cisternae, the TGN of the other eukaryotic cells.

The purpose of the present study was to detect the release of the ESV in order to solve some open questions: (1) if exocytosis occurs and (2) if the secretory granules fragment before their release on cell surface.

2. Materials and methods

2.1. Organisms and culture of trophozoites

*Giardia lamblia* strain WB (American Type Culture Collection, No. 30957) was cultivated in TYI-S-33 medium enriched with 10% heat-inactivated fetal bovine serum [10] at pH 7.1, without added vitamins, iron, or antibiotics [3], but supplemented with 0.1% bovine bile [11] for 48–72 h, at 37 °C.

2.2. Induction of encystation

Encystation was induced as described previously [8]. Briefly, pre-encysting cultures were grown to late log phase for 48 h in TYI-S-33 medium (pH 7.05) without bile, and without antibiotics. Encystation was initiated by removing the spent medium and non-adherent cells and refeeding the adherent cells with encystation medium: TYI-S-33 without bovine bile, adjusted to pH 7.8, and supplemented with 0.25 mg/ml porcine bile and 5 mM lactic acid.

2.3. Transmission electron microscopy

Cells were fixed in situ, without chilling or washes, overnight, at room temperature, in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After that, the cells were gently scraped off with rubber-police men and washed in phosphate buffer saline (PBS). Post-fixation was performed for 15 min in 1% OsO4 in cacodylate buffer containing 5 mM CaCl2 and 0.8% potassium ferricyanide. Cells were washed in PBS, dehydrated in acetone and embedded in Epon. Ultra-thin (50 nm), semi-thin sections (100 nm) and serial thin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 1210 transmission electron microscope.

2.4. Scanning electron microscopy

Living cells were adhered to poly-L-lysine-coated glass coverslips, fixed with 2.5% glutaraldehyde in cacodylate buffer, post-fixed for 5 min in 1% OsO4, dehydrated in ethanol, critical point dried with CO2, sputter-coated with gold-palladium and examined in a Jeol 5800 scanning electron microscope operating at 12 KV.

2.5. Freeze-fracture

After overnight fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, the cells were washed twice in PBS and then exposed to increasing concentrations of glycerol in cacodylate buffer until a final concentration of 30% glycerol was attained. Specimens were mounted on Balzers support disks and rapidly frozen in the liquid phase of Freon 22 cooled by liquid nitrogen and immediately transferred to liquid nitrogen. Subsequently, the specimens were freeze-fractured at −115 °C in a Balzers BAF 300 freeze-fracture machine and immediately shadowed with platinum/carbon at $2 \times 10^{-6}$ Torr. Replicas were recovered in distilled water, cleaned with sulfuric acid and/or sodium hypochloride, mounted on 200-mesh grids, and examined in a Jeol 1210 transmission electron microscope.
2.6. Cytochemistry

2.6.1. Thiéry’s technique (periodic acid–thiosemicarbazide–silver proteinate) [12]

Ultrathin sections of cells fixed in glutaraldehyde and OsO₄, as above described, were collected on gold grids and treated for 20 min with 1% periodic acid. After rinsing in distilled water the sections were incubated for 48–72 h in aqueous solution containing 1% thiosemicarbazide, and 10% acetic acid. After rinsing sequentially with 10%, 5%, and 1% acetic acid and distilled water, the sections were exposed to 1% silver proteinate for 30 min in the dark, at room temperature. Sections were observed unstained. Controls were performed by omission of periodic acid step.

3. Results

*Giardia lamblia* exists in two morphologically and metabolically distinct forms: the trophozoite and the cyst. We induced cultured *G. lamblia* trophozoites to encyst in vitro following the protocol established by Gillin et al. [3]. These authors demonstrated that when exposed to encystation stimuli (elevated pH and bile),
G. lamblia developed vesicles (ESV), which are not normally observed in non-encysting trophozoites. The ESVs are osmiophilic, prominent vesicles (Figs. 1, 2(a) and (b)). Until the present work, the ESV content release has not been documented. We have screened many encysting cells, and the better images were obtained in 24 h encysting cells, where we were able to observe the exocytic process both by transmission electron microscopy (TEM) (Figs. 2(a) and (b); 3) and scanning electron microscopy (SEM) (Figs. 4(a) and (b)). We took advantage of well-fixed cells analyzed by TEM and SEM, and a careful screening looking for exocytosis. No drugs or stimulus were used. In addition, frozen cells processed for freeze-fracture were also studied (Fig. 3). Large granules, similar in electrondensity, size and shape were observed in process of docking and fusion with the plasma membrane (Figs. 2(a), (b) and (f); 3; 4(a) and (b)). However, incomplete fusion seemed to occur, since membrane fragments were observed during the vesicle release (Figs. 2(a) and (b)). These membranes seem to be the remnants of an exocytosed ESV. In addition, empty vesicles, here termed membrane ghosts were observed either free in the extracellular medium (Fig. 2(c)) or attached on the flagellar surface (Figs. 2(d) and 4(c)), suggesting a sealing membrane process. These ghosts were positive for carbohydrates when the Thiéry technique [12] was used (Fig. 2(c)), suggesting an inside-in membrane sealing. After granule discharge, the plasma membrane appeared in process of healing (Fig. 2(e)).

One interesting observation was that, even during the cyst wall formation, endocytosis takes place (Fig. 2(f)), once empty vesicles were observed in process of internalization. This process could also represent exocytic images. However the plasma membrane is well delimited, and vacuoles containing a large amount of similar structures were observed (Fig. 5). These observations were also made when freeze-fracture was performed (Fig. 3).

By SEM, the encysting Giardia was seen in process of rounding-up (Fig. 4(a)). The cells surface presented several blebs and cup-like structures, some of which exhibited granules in process of discharge (Figs. 4(a) and (b)). The externalized flagella were invariably covered by several membrane vesicles (Fig. 4(c)), which seem to represent empty vesicles, as seen by TEM (Fig. 2(d)). A cartoon representative of the process is shown in Fig. 6.
4. Discussion

Encystation of *G. lamblia* is required for survival outside the host, as well as for initiation of new infections. Biogenesis of a protective cyst wall is a key mechanism for the continuity of infections caused by *G. lamblia*. The processing of the cell wall is a complex series of coordinated events involving components of the endomembrane system, likewise the activation of wall synthesis, including the intracellular packaging and transport of wall macromolecules, their secretion, and extracellular assembly.

Reiner et al. [13] demonstrated that during encystation trophozoites develop prominent vesicles named ESV. They also showed that cysts antigens were concentrated in these vesicles early in encystation, and observed in the cyst wall later in differentiation, thus supporting the idea that ESV comprise a regulated pathway for transport of cyst wall components to nascent wall.

An important point to understanding the ESV as dense granules-like secretory vesicles, is the elucidation of their fate during secretion. The key criterion for secretory vesicles is their ability to fuse with plasma membrane and thus release their content. However, this process appears to be completed so quickly, that it has not been previously documented. Reiner et al. [4] showed a frozen section micrograph of a cell exposed to encystation medium for 22 h, demonstrating an early stage in exocytosis before the ESV contents have been released. These authors proposed that the vesicle contents are released to the nascent cyst wall by exocytosis, since they observed contiguity between the vesicle membrane and the plasma membrane. They also suggested an additional layer of membrane partly surrounding the ESV, which could be originated from ER membrane [4]. Continuity of the ESV membrane with the plasma membrane of the cell suggested that the contents of the vesicle could be released by exocytosis. After this single observation, no additional detection of vesicle release has been published. It was suggested [14] that release of mature ESV contents by exocytosis appears to be a continuous process, dependent on maturation rather than elicited by a external trigger.

Little is known about the molecular and cellular mechanisms by which cyst wall materials are released and deposited extracellularly. Several explanations have been proposed to clarify the mechanisms involved in vesicle release and cyst wall formation [5–7]. ESV docked in place under the cell membrane was observed previously, but not its content release [4]. This last group has indicated that cyst wall components are released by exocytosis. However, the way in which the release occurs is still undefined. The difficulties encountered by others in observing vesicle release by electron microscopy may be due to the rapidity and/or synchrony of this process. Therefore the stimulus for vesicle release remains to be determined. On the other hand, if vesicles fuse upon contact with plasma membrane, why does the cell not increase its size? Does a rapid mechanism of membrane recycling take place during or after exocytosis of the ESV? Do the ESVs mature directly to become a secretory granule and directly fuse with plasma membrane or fragment into secretory smaller vesicles destined for the plasma membrane? Marti and Hehl [9] proposed a complete dispersal of ESV into small secretory vesicles before secretion of cyst wall material, suggesting that the ESV could correspond to Golgi cisternae, and when are dispersed could represent the TGN of the other eukaryotic cells. Here, we show for the first time, that the large ESVs are not fragmented before fusing with the plasma membrane, and exocytosis occurs by a different process in which an incomplete membrane fusion occurs.

During the process of vesicle release, a typical exocytosis does not seem to occur, since during membrane

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Fig. 6. Cartoon representative of the granule release in encysting *G. lamblia*. In (a)–(b) the encysting granule (ESV) is seen in process of docking; in (c) incomplete fusion with the plasma membrane (PM) is observed; in (d) membrane fragments are formed during the vesicle release; in (e) these membranes remnants are sealed and empty vesicles are formed, here termed membrane ghosts which become free in the extracellular medium and/or attached to flagella.
fusion, some membranous segments appeared to be disrupted and released to extracellular medium. If the membrane ghosts are originated from the enveloping vesicle membrane or from the plasma membrane needs to be determined. We propose that the membranous fragments reseal forming empty vesicles, which are found in the extracellular medium. The plasma membrane also seems to be restored. Empty vesicles are here considered as membrane “ghosts” or empty vesicles. These membranes appear to be inside-in since they are labeled on their external surface by Thiéry’s technique, which detects carbohydrates [12]. Many of these ghosts attach on the flagellar membranes, thus decorating the externalized flagella. Recently, Arguello-Garcia et al. [15] have studied the carbohydrate-rich moieties and the fibrillate layer of early encysting Giardia cells. These authors showed that cyst wall formation in G. lamblia involves a stepwise pattern and using ruthenium red they detected the extracellular assembly of cyst wall [15]. Our observations using Thiéry technique are in agreement with their results.

These empty vesicles can be those previously described in the peritrophic spaces of mature cysts [16] and also in excysting organisms [17]. Coggins and Schaefer [18] observed in G. muris a large number of smaller, membrane bound vesicles on the outer surface of the cell membrane in excysting parasites. These authors reported that these vesicles varied greatly in size and appeared to be formed through exocytosis. Also, Buchel et al. [19] have observed microspherules bordering the area of the cyst wall opening, and Hetusko et al. [16] described that excysting cells were sometimes surrounded by clusters of vesicles. All these cluster of vesicles are similar to the sealed membranes fragments herein observed during the process of ESV release.

The apparent absence of a classical Golgi makes Giardia an interesting model in which primordial mechanism can be involved in protein transport and secretion. The regulated nature of cyst wall material secretion, together with a unique secretory system implicates in novel insights in the process of exocytosis and evolution. These TEM observations lead us to suggest that a complete membrane fusion does not occur. This would represent an unusual mechanism of secretion.

We propose that:

1. During the ESV exocytosis, the process of fusion is incomplete and disrupted membranes are formed.
2. These membranous fragments reseal and form empty vesicles (ghosts).
3. These vesicles may be (a) endocyted, (b) adhered on the flagellar surfaces, or/and (c) located in the peritrophic spaces.

This proposal could answer several open questions raised in the literature [20], such as (1) the origin of the observed vesicles in cysts, (2) why the cell does not increase in size during the exocytosis of the ESVs, (3) the probable mechanism of membrane recycling after the release of ESV (endocytosis), (4) from where come the vesicles observed over the flagella during the encystation process.

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