Autophagy is involved in starvation response and cell death in *Blastocystis*

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Previous studies have demonstrated that colony forms of *Blastocystis* undergo cell death with numerous membrane-bound vesicles containing organelles located within the central vacuole, resembling morphological features of autophagy. In this study, we investigated whether *Blastocystis* underwent autophagy upon amino acid starvation and rapamycin treatment. Concurrently, we provide new insight into a possible function of the central vacuole. The use of the autophagy marker monodansylcadaverine, and the autophagy inhibitors 3-methyladenine and wortmannin, showed the existence of autophagy in amino-acid-starved and rapamycin-treated *Blastocystis*. Confocal microscopy and transmission electron microscopy studies also showed morphological changes that were suggestive of autophagy. The unusually large size of the autophagic compartments within the parasite central vacuole was found to be unique in *Blastocystis*. In addition, autophagy was found to be triggered when cells were exposed to the cytotoxic antibody mAb 1D5, and autophagy was intensified in the presence of the caspase inhibitor zVAD.fmk. Taken together, our results suggest that the core machinery for autophagy is conserved in *Blastocystis*, and that it plays an important role in the starvation response and cell death of the parasite.

INTRODUCTION

Autophagy, in particular macroautophagy, is the major mechanism used by eukaryotic cells to degrade long-lived proteins, and it is perhaps the only known pathway for degrading organelles (Levine & Klionsky, 2004). It is believed to be a conserved process in all eukaryotic cells. During autophagy, a double-membrane structure, known as the phagophore, forms and expands to sequester a portion of cytoplasm in the form of an autophagosome. The autophagosome fuses with a lytic compartment, the engulfed materials are degraded, and the resulting macromolecules are recycled (Klionsky & Emr, 2000; Levine & Klionsky, 2004). In yeast, autophagy is induced under adverse conditions, such as nutrient deficiency, to degrade proteins that are not needed, and recycle the amino acids for survival (Takeshige et al., 1992; Yorimitsu & Klionsky, 2005). In mammals, autophagy has been implicated in development, differentiation and disease processes (Kuma et al., 2004; Levine & Klionsky, 2004; Shintani & Klionsky, 2004).

In contrast to the extensive studies of autophagy in yeast and higher eukaryotes, the mechanism and significance of autophagy in the more primitive protozoan parasites is far less well understood. Recent reports have indicated an increasing interest in this field. Autophagy has been observed in dying *Tetrahymena thermophila* cells following staurosporine treatment (Christensen et al., 1998). In *Leishmania donovani*, treatment with antimicrobial peptides induces cell death via autophagy (Bera et al., 2003). Autophagy has been found to be essential for differentiation and virulence of *Leishmania major*, and cysteine proteases have been found to be necessary for the process (Besteiro et al., 2006; Williams et al., 2006). In *Trypanosoma*, candidate autophagy genes have been identified through a bioinformatic search (Herman et al., 2006), and some components of the Atg8 conjugation system have been verified to function in differentiation of *Trypanosoma cruzi* (Alvarez et al., 2008a, b). Recently, autophagy has been found to play an important role in the proliferation and differentiation of the enteric protozoan parasite *Entamoeba* (Piczarri et al., 2008).

*Blastocystis* is a protozoan parasite that inhabits the large intestines of humans and many other animals (Tan, 2004, 2008). The parasite is prevalent in developing countries, and it has been associated with intestinal diseases. It is a
polymorphic organism with four commonly described forms: vacuolar, granular, amoeboid and cystic. The vacuolar form is commonly seen in the stools of patients with intestinal disorders, and this form is the predominant cell type seen in axenized in vitro cultures. In this form of Blastocystis, a thin rim of cytoplasm surrounds a characteristic large central vacuole that takes up to 90% of the cell volume (Tan, 2008). However, little is known about the function of the enigmatic central vacuole (Tan, 2004).

Although there has been no report of autophagy in Blastocystis, previous studies have shown unexplained morphological features suggestive of autophagy. For example, ageing Blastocystis cells, grown as colonies, have been found to have cytoplasmic vacuolation or membrane-bound vesicles containing organelles within the central vacuole (Tan et al., 2001). These observations have been seen mostly in cells located in the centre of the colony. As these cells have limited access to nutrients compared with cells at the periphery, it is likely that the autophagic machinery has been triggered in response to nutrient deprivation (Tan et al., 2001; Tan & Nasirudeen, 2005).

Given the importance of autophagy in the wide array of host–pathogen interactions (Alvarez et al., 2008b; Colombo, 2007), we aimed to verify the existence of autophagy in Blastocystis, and elucidate the role of autophagy in the survival and proliferation of this parasite. Furthermore, the study of Blastocystis autophagy may shed light on the function of the unique central vacuole of this parasite.

**METHODS**

**Culture of organism.** Blastocystis subtype 7 (formerly known as Blastocystis hominis isolate B) was obtained from a local patient, and maintained axenically in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% inactivated horse serum, as described (Ho et al., 1993). Cells were grown anaerobically using an AnaeroJar (Nunc) after they were removed by a sterile inoculating loop, and stained with 0.05 mM MDC in PBS was added to the suspension, and it was incubated at 37 °C for 15 min. After incubation, the cells were washed three times in PBS, and mounted on a glass slide under a coverslip. Cells were then visualized with a fluorescence microscope (Olympus BX60) using an excitation filter of 360 nm and an emission filter of 525 nm. The images were captured by a CCD camera (Olympus DP70). The percentage of MDC-positive cells was determined by scoring 500 cells per group.

To stain colony cultures of Blastocystis, colonies embedded in the agar were removed by a sterile inoculating loop, and stained with 0.05 mM MDC in PBS for 30 min. Then the colonies were washed twice in 1 ml PBS, with a 5 min interval between each wash. The colonies were then placed on a slide, and pressed gently with a coverslip. The colonies were viewed under a fluorescence microscope to examine the uptake of the stain by the cells at the centre and at the periphery of the colony.

**Effect of the autophagy inhibitors 3-methyladenine and wortmannin.** To test the effect of the autophagy inhibitors 3-methyladenine (3-MA) and wortmannin on Blastocystis, cells were pretreated with 20 mM 3-MA (Sigma) or 50 μM wortmannin, and they were incubated for 3 h prior to amino acid starvation, rapamycin treatment or exposure to mAb 1D5, as described above.

Western blotting. Blastocystis cells were incubated in IMDM medium or HBSS for 1–3 h. Cells were lysed in buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 20 mM β-glycerophosphate, 1 mM Na3VO4, and Roche complete protease inhibitor), and the soluble fraction was obtained by centrifugation (14,500 g, 10 min) at 4 °C. The total protein concentration was determined by the Bradford assay. The total protein (50 μg) was electrophoresed on a 12% SDS-PAGE gel, and transferred to a PVDF membrane (GE Healthcare) by semi-dry blotting (Bio-Rad). The membrane was then blocked with 5% skim milk in PBS-T (1 × PBS with 0.1% Tween-20), incubated with rabbit anti-human microtubule-associated protein light chain 3β (MAP LC3β) polyclonal antibody (dilution 1:500; Santa Cruz Biotechnology), washed in PBS-T and incubated in a 1:10000 dilution of horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology). The proteins were detected by chemiluminescence (ECL plus; GE Healthcare) and exposed to an X-ray film (Kodak).

**BLAST searches of ATG orthologues in Blastocystis.** Bioinformatic analysis was carried out to search for orthologues of Autophagy-related (ATG) genes in Blastocystis. To date, 32 ATG genes have been identified in Saccharomyces cerevisiae and other fungi (Kanki et al., 2009; Okamoto et al., 2009). Protein sequences of the yeast ATGs were retrieved from Swiss-Prot (www.ebi.ac.uk/uniprot/), and used as queries to perform a BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/blast.cgi) search against the Blastocystis EST database (http://blastdb.bcm.umontreal.ca/searches/organism.php?orgID=8) stored locally using StandAlone BLAST version 2.2.18.
Confocal microscopy examination of MDC and Lysotracker Red costaining. Following various treatments, the cell pellet was stained with 0.05 mM MDC for 15 min at 37 °C, and then with 50 nM Lysotracker Red DND-99 (Molecular Probes) for a further 30 min at 37 °C. After incubation, the cells were washed three times in PBS, and mounted on glass slides under coverslips. Fluorescent images were obtained by using a confocal microscope (Olympus Fluoview FV500). MDC was excited at 405 nm, and the emission was collected at 505–525 nm. Lysotracker Red was excited at 543 nm, and the emission was collected at 584–654 nm.

Transmission electron microscopy. The ultrastructural features of Blastocystis cells under various treatments were examined using transmission electron microscopy (TEM). Briefly, cells were fixed with 2 % glutaraldehyde and 2 % paraformaldehyde in PBS for 3 h at 4 °C. The cells were then pelleted at 1000 g for 5 min, and washed twice in PBS, and twice in deionized water. After each washing step, cells were spun at 1500 g, and the supernatant was discarded. Cells were then post-fixed for 2 h with 1 % osmium tetroxide containing 1 % potassium ferricyanide at room temperature, followed by dehydration with a graded ethanol series, and infiltration and embedding with LR-White resin (London Resin Company). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using an EM208S transmission electron microscope (Philips).

Statistical analysis. Experiments were repeated at least twice. Quantitative data were statistically evaluated using Student’s t test, and differences were considered significant at the level of P<0.05. Values are given as means ± SD.

RESULTS

MDC accumulates in the central region of the Blastocystis colony

MDC is an autofluorescent compound that has been reported to specifically label autophagic vacuoles in in vivo and in vitro conditions because of its ability to act as a lysosomotropic agent and as a solvent polarity probe (Biederbick et al., 1995; Munafò & Colombo, 2001; Niemann et al., 2000). Since the ultrastructural features of Blastocystis colonies have implied the occurrence of autophagy, MDC labelling was performed in Blastocystis colonies. After 10 days of incubation, discrete buff-coloured opaque colonies were observed macroscopically in agar inoculated with Blastocystis cells. Using an inverted microscope, distinct biconvex-disc-shaped colonies embedded in the soft agar were observed. Individual colonies without compromised integrity were stained with MDC to examine the uptake of the stain by the cells at the centre and the periphery of the colony (Fig. 1). While a certain level of background staining was seen in all cells, true MDC-positive staining was identified by the prominent fluorescence intensity. It was observed that cells located in the centre of the colony showed a significant proportion of MDC-positive cells, whereas cells located at the periphery of the colony showed virtually no MDC-positive staining. The data suggest that autophagy may indeed be triggered in cells located at the centre of the colony.

MDC incorporation increases with amino acid starvation and rapamycin treatment in Blastocystis

Because it has been shown in other eukaryotic cells that autophagy is often rapidly upregulated when cells are under nutritional stress (Levine & Klionsky, 2004; Munafò & Colombo, 2001; Takeshige et al., 1992), Blastocystis was deprived of amino acids to further study and ascertain the autophagy phenomenon in this organism.

Blastocystis cells were incubated in IMDM medium (control) or in HBSS for 1–3 h. After treatment, cells were stained with MDC (Fig. 2), and the MDC-positive cells were scored. The percentage of MDC-positive cells increased from 6.7 ± 0.9 % in cells incubated in IMDM medium to 19.7 ± 2.2 % (P<0.05) after 1 h amino acid starvation. There was also a time-dependent increase of MDC-positive cells as the starvation progressed to 2 h (28.6 ± 0.2 %; P<0.05) and 3 h (38.0 ± 1.4 %; P<0.05) periods. Hence, these results suggested that autophagy was rapidly triggered in Blastocystis cells in response to amino acid starvation.

Fig. 1. MDC staining of colony forms of Blastocystis. Blastocystis cells were grown as colonies in soft agar. On day 10, intact colonies embedded in the agar were removed by using an inoculating loop, stained with 0.05 mM MDC, and viewed under a fluorescence microscope. Arrows indicate representative MDC-positive cells.
Target of rapamycin (TOR) is a conserved Ser/Thr kinase, and is a central controller of cell growth. It is a negative regulator of autophagy, and inhibition of TOR leads to induction of autophagy (Meijer & Codogno, 2004). Nutrient starvation or absence of growth factors can inhibit TOR, and trigger autophagy in yeast and mammalian cells (Lum et al., 2005; Wullschleger et al., 2006). It is very likely that the TOR signalling pathway also exists and functions in Blastocystis, and that the accumulation of MDC staining observed in serum-starved Blastocystis cells was due to the inactivation of TOR. To verify the existence of the TOR signalling network in Blastocystis, we investigated whether rapamycin, the prototypical inhibitor of TOR, could induce autophagy in Blastocystis.

Blastocystis cells were treated with 100, 500 or 1000 nM rapamycin for a period of 3 h. MDC staining was visually scored using fluorescence microscopy (Fig. 3). Similar to amino acid starvation, punctuate intensive staining by MDC was observed in rapamycin-treated cells. As compared with the DMSO control (8.0 ± 1.0 % MDC-positive cells), cells that had been incubated with 1000 nM rapamycin had a significantly higher percentage of MDC-positive cells (45.5 ± 12.4 %; \( P<0.05 \)). Rapamycin at a concentration of 100 or 500 nM did not induce a significant change in the number of MDC-positive cells: 12.0 ± 4.5 % and 14.2 ± 5.6 %, respectively. These results indicate that rapamycin induced autophagy in Blastocystis, and thus the TOR signalling pathway is likely to be conserved in Blastocystis. Trypanosome TOR has been reported to have an IC\(_{50}\) of 152 nM for rapamycin after treatment for 72 h (Barquilla et al., 2008). The fact that Blastocystis was insensitive to rapamycin concentrations below 1000 nM may be due to the brief incubation period of 3 h. Supplementary Fig. S2 shows that after incubation with rapamycin for 72 h, Blastocystis exhibited a dose-dependent increase in the percentage of MDC-positive cells at rapamycin concentrations of 100, 500 and 1000 nM.
**Inhibitory effects of 3-MA and wortmannin on MDC incorporation**

To evaluate whether the incorporation of MDC was indeed dependent on autophagy, cells were pretreated with 3-MA or wortmannin, which are inhibitors of autophagic sequestration. 3-MA and wortmannin are phosphatidyl-inositol 3-kinase (PI3K) inhibitors, and they inhibit both class I and class III PI3K. Class I PI3K generates products that inhibit autophagic sequestration, while class III PI3K products stimulate autophagic sequestration downstream of class I enzymes; therefore, the overall effect of 3-MA and wortmannin is to block autophagy (Blommaart et al., 1997; Petiot et al., 2000). The addition of 3-MA to 3 h amino-acid-starved cells decreased the percentage of MDC-positive cells from 38.0 ± 1.4 % to 9.5 ± 0.6 % (P<0.05 versus control consisting of Blastocystis cells in IMDM medium), and the addition of wortmannin decreased the percentage to 5.5 ± 3.1 % (Fig. 2). A similar inhibitory effect was seen in rapamycin treatment, as the MDC uptake of cells treated with 1000 nM rapamycin dropped from 45.5 ± 12.4 % to 8.14 ± 0.6 % and 8.7 ± 1.6 % upon the addition of 3-MA and wortmannin, respectively (Fig. 3). Therefore, in the presence of 3-MA or wortmannin, there was an inhibitory effect on the incorporation of MDC in both amino-acid-starved and rapamycin-treated Blastocystis cells.

**Homology search of ATG genes, and detection of LC3-like antigen in Blastocystis**

BLAST searches for Atg orthologues in the Blastocystis EST database generally resulted in very low scores (Supplementary Table S1), which may be due to the incompleteness of the database. Microtubule-associated protein light chain 3 (LC3) or Atg 8 homologues are widely used to monitor autophagy in various organisms including protozoan parasites (Alvarez et al., 2008a; Klionsky et al., 2008). However, no significant match was found in a BLAST search of the Blastocystis EST database (Table S1). Using a rabbit polyclonal antibody raised against amino acids 1–50 mapping at the N terminus of human MAP LC3β, we identified a LC3-like antigen in Blastocystis cell lysates (Supplementary Fig. S1). The size of the LC3-like antigen in Blastocystis was 20 kDa, which is slightly higher than that of the mammalian homologue of LC3-I (16 kDa) (Mizushima, 2004). Interestingly, the LC3-like antigen of Blastocystis underwent a time-dependent degradation when cells were under amino acid starvation conditions for different periods of time.

**MDC staining colocalizes with Lysotracker Red staining in the central vacuole**

It was observed that most of the MDC staining accumulated in the central vacuole. To elucidate the function of the Blastocystis central vacuole in autophagy, morphological changes of amino-acid-starved and rapamycin-treated cells were assessed via confocal microscopy after co-staining with MDC and Lysotracker Red. Lysotracker Red is a fluorophore that selectively accumulates in acidic compartments, such as lysosomes, whereas MDC was first described as a specific marker of autphagic vacuoles (Biederbick et al., 1995). By double staining, the dynamics of the autophagy process were anticipated because early autophagosomes should be stained with MDC only, and after their fusion with lysosomes co-localization can be observed. However, Fig. 4 shows that MDC and Lysotracker Red staining almost always co-localized. MDC or Lysotracker Red single-labelled controls were checked to ensure no cross-talk or bleed-through of the two fluorescent dyes (results not shown). The results were not surprising, since the specificity of MDC staining has been cautioned by other workers, and it has been suggested that MDC is similar to other acidotropic dyes (eg, Lysotracker Red), and that it may preferentially label later stages in the degradation process of autophagy. Early autophagosomes may not be readily labelled by MDC because they are not acidic (Bampton et al., 2005; Klionsky et al., 2008; Mizushima, 2004). Nonetheless, these results suggest that, in Blastocystis, MDC and Lysotracker Red label the same autophagic compartments of acidic pH, although the detailed nature of the structure awaits further investigation.

Examinations of the confocal microscopy images revealed very unique MDC staining patterns in Blastocystis. Most of the extensive staining was seen inside the central vacuole as either individual circular specks or irregular-shaped objects, which were likely to be clusters of several specks. The size of MDC-labelled individual specks was 0.5–1 μm, which is comparable to the size of autophagosomes in yeast (0.3–0.9 μm) (Yorimitsu & Klionsky, 2005). However, the size of the irregular-shaped objects was 2–8 μm, and this has not be found in yeast or mammalian cells, except for the 5–10 μm autophagosomes containing bacteria or protozoan parasites (Andrade et al., 2006; Nakagawa et al., 2004). Interestingly, in another enteric protozoan parasite, Entamoeba invadens, the sizes of autophagosome-like structures were very similar to those we found in Blastocystis (Picazarrri et al., 2008). In that study, the size of Atg8-associated structures varied from <1 μm to >4 μm, and the appearance of the large-sized structures coincided with the initiation of encystation process.

**Ultrastructural features of Blastocystis under nutrient stress**

Amino acid-starved or rapamycin-treated Blastocystis cells displayed ultrastructural features resembling those of autophagy (Fig. 5). As compared with the untreated control (Fig. 5a, b), most of the treated cells showed cytoplasmic vacuoles with membranous inclusions, and numerous vacuoles were observed to be accumulating within the central vacuole. The content of the autophagic-like vacuoles included intact mitochondria, membrane
whirls and amorphous materials. The presence of vacuoles containing membranous inclusions or intact organelles is highly suggestive of autophagy. The size of most of the autophagic vacuoles observed in TEM was 0.5–1 μm, while some were 2 μm (Fig. 5i), 4 μm (Fig. 6a) and 6 μm (Fig. 5i arrow) in size. This is consistent with the size of MDC-labelled structures observed by confocal microscopy. Less frequently, it was observed that cytoplasmic contents were invaginating through vesicle-like or thin-tube-like membrane structures into the central vacuole (Fig. 6).

Interestingly, there were vesicles budding at the end of the filament-like structures. We hypothesize that the autophagic vacuoles were formed in different ways: membrane expansion, and sequestration of cytoplasmic material in the cytoplasm (Fig. 5j); extensive vacuolation in cytoplasmic regions near to the central vacuole, and formation of vesicles that invaginate into the central vacuole (Fig. 6a–d); formation of a thin-tube-like structure that extrudes into the central vacuole, and budding-off vesicles at the end of the tubular invagination into the central vacuole (Fig. 6e–g). We posit that the central vacuole is a reservoir for most autophagic vacuoles. Lysosomes are likely to be translocated from the cytoplasm into the central vacuole in the meantime, and fuse with newly formed autophagic vacuoles (Fig. 5e, f). Our observation that some autophagic vacuoles displayed ultrastructural evidence for double membranes characteristic of such structures, surrounded by the central vacuole membrane, seems to support this model (Fig. 5g, i).

**mAb 1D5 induces autophagic features in Blastocystis**

Previous studies have demonstrated that the cytotoxic monoclonal antibody mAb 1D5 induces apoptotic features in *Blastocystis*; these features included externalization of plasma membrane phosphatidylserine, mitochondrial outer-membrane permeabilization, and DNA fragmentation (Nasirudeen & Tan, 2004; Nasirudeen et al., 2001). While apoptotic features can be inhibited by the caspase...
inhibitor zVAD.fmk, and the mitochondrial outer-membrane permeability (MOMP) inhibitor cyclosporin A, the cells cannot be rescued from death (Nasirudeen & Tan, 2005). It has been suggested that mAb 1D5 can elicit a programmed-cell-death response in *Blastocystis* that is independent of caspases, mitochondria, or both, probably through an alternative pathway other than apoptosis (Nasirudeen & Tan, 2005; Tan & Nasirudeen, 2005).

Since autophagic cell death has often been triggered as an alternative cell death pathway when apoptosis is blocked (Gozuacik & Kimchi, 2004), it was of interest to investigate whether mAb 1D5 could induce autophagic cell death in *Blastocystis*.

In order to evaluate the possibility of autophagic cell death in *Blastocystis* treated with mAb 1D5, alone and in the presence of zVAD.fmk and/or cyclosporin A, cells were labelled with MDC. The cells in each treatment were visually scored for MDC-positive staining by fluorescence microscopy (Fig. 7). Cells treated with growth medium or a

**Fig. 5.** Ultrastructural observations of autophagic vacuoles deposited in the central vacuole of *Blastocystis*. Untreated controls (a, b) displayed normal cell morphology. The central vacuole contains flocculent contents. Nu, nucleus; M, mitochondrion; G, Golgi apparatus; L, lipid inclusion; CV, central vacuole. Amino-acid-starved cells (c–j) and rapamycin-treated cells (k–n) showed numerous autophagic-like vacuoles within the central vacuole. (f) A mitochondrion appears to be in the process of fusion with a lysosome [enlarged from the upper box in (e)]. (g) Several double-membrane vesicles with more disintegrated contents [enlarged from the lower box in (e)]; the inset shows a region with three layers of membranes. (i) A vesicle containing two mitochondria and cytoplasm (arrow), a vacuole containing membranous whirls (dashed arrow and inset), and another double-membrane vacuole (arrowhead) [enlarged from (h)]. (j) A double membrane formed in the cytoplasm (arrow) and a cytoplasmic vacuole (dashed arrow) [enlarged from (h)]. (l) A vacuole with a mitochondrion and some cytoplasmic material [enlarged from (k)]. (n) Two vesicles with an inner limiting membrane (arrows), and another vesicle containing enclosed membrane sacs and a lipid granule (dashed arrow) [enlarged from the box in (m)]. Bars, 2 μm (a, b, h), 1 μm (c, d, e, i, j, k, m), 0.5 μm (f, g, l, n).
non-specific IgM monoclonal antibody, alone or in the presence of zVAD.fmk and/or cyclosporin A, showed a small percentage of MDC-positive cells (<15%). In contrast, mAb-1D5-treated cells had a higher percentage (18.9%) of MDC-positive cells compared with the control. In the presence of pan-caspase inhibitor zVAD.fmk, 37.9% of mAb-1D5-treated cells showed MDC positivity. However, when cells were exposed to mAb 1D5 in the presence of cyclosporin A, the percentage of MDC-positive cells decreased to 11.6%. mAb-1D5-treated *Blastocystis* pre-exposed to zVAD.fmk and cyclosporin A showed 10.8% MDC-positive cells. The autophagy inhibitor 3-MA was also found to have an inhibitory effect on the incorporation of MDC with mAb 1D5 treatment. These results indicate that the phenomenon of autophagy was triggered by mAb 1D5 treatment, and was intensified in the presence of zVAD.fmk. In addition, the mAb-1D5-elicited autophagy might be associated with the induction of MOMP.

**DISCUSSION**

In this study, we present what is believed to be the first description of autophagy in the protozoan parasite *Blastocystis* through MDC labelling and ultrastructural characterization. Strong and punctate MDC staining was observed in cells located in the centre of a *Blastocystis* colony. Within a colony, the cells in the centre have less access to nutrients and, hence, they are most likely to undergo starvation, as compared with the cells at the periphery of the colony. In this study, cells grown in liquid culture and deprived of amino acids also had a high percentage of MDC-positive staining. In addition, time-dependent increase of MDC staining was observed during the first 3 h of amino acid starvation. These results suggest a strong correlation between MDC staining and the nutritional state of the cell. Since MDC has been reported to stain autophagic vacuoles, and autophagy is rapidly upregulated in response to nutrient-deficient conditions in other organisms (Takeshige *et al.*, 1992; Yorimitsu & Klionsky, 2005), the intensive MDC staining should be a good reflection of the autophagic activity in *Blastocystis*, and it was used in further investigation. The results also demonstrated that autophagy occurs naturally when cells have limited access to nutrients, and that it can be rapidly induced by removing nutrients from the culture medium.

Results of the current study suggest that some of the autophagy machinery is conserved in *Blastocystis*. TOR is a conserved Ser/Thr kinase, and a negative regulator of autophagy. Rapamycin induces autophagy through inhibition of TOR (Meijer & Codogno, 2004). It was found that, in *Blastocystis*, treatment with rapamycin elicited a similar response to that of amino acid starvation. Rapamycin treatment was effective in intensifying MDC-labelled vesicles; hence, *Blastocystis* is likely to have a homologue of TOR, and the TOR signalling pathway should also exist and function in *Blastocystis*. The PI3K inhibitors 3-MA and

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**Fig. 6.** Ultrastructural observations of cytoplasmic invagination into the central vacuole when cells were deprived of amino acids (a, b and e), or treated with rapamycin (c, d, f and g). (a–d) Invagination of membrane-bound vesicles into the central vacuole. Multiple vacuolations were seen in the cytoplasmic region near the central vacuole, and some vesicles appeared to be in the process of invaginating into the central vacuole. (e–g) Invagination of membrane-bound filaments into the central vacuole, with vesicles located at the tip of the filament (arrows). Bars, 2 µm (a, c, f, g), 1 µm (b, d, e).
wortmannin inhibited the incorporation of MDC effectively. Both class I and class III PI3K have been reported to regulate autophagy in yeast and mammalian cells. Class I PI3K generates products to inhibit autophagic sequestration, while class III PI3K products stimulate autophagic sequestration downstream of class I enzymes, so the overall effect of 3-MA and wortmannin is to block autophagy (Blommaart et al., 1997; Petiot et al., 2000). Inhibition of MDC incorporation by 3-MA and wortmannin suggests that a similar regulation pathway exists in Blastocystis, and also confirms the autophagic nature of MDC-labelled vesicles. An LC3-like antigen was found via cross-reactivity of anti-human LC3 antibody. In other studies with mammalian cells, upon initiation of macroautophagy, the cytosolic form LC3-I is conjugated with phosphatidylethanolamine, and becomes the membrane-bound form LC3-II, which is specifically located on isolation membranes and autophagosomes (Klionsky et al., 2008; Mizushima, 2004). Immunoblotting of LC3 upon autophagy induction will thus show the conversion of LC3-I to LC3-II. However, the current study detected only one reactive band using anti-human antibody. The possibility of the antibody binding to an unrelated protein that shares the same epitopes as LC3 cannot be excluded, and the identity of this LC3-like antigen needs to be confirmed in a future study. Nonetheless, the degradation of LC3-like antigen may reflect the non-selective degradation of cellular proteins during the autophagy process.

Morphological examination by confocal microscopy and TEM revealed several unique features of autophagy in Blastocystis. Most of the extensive MDC staining was observed inside the central vacuole, and this suggests a role of this organelle in the autophagy process. MDC-stained structures appeared as either individual circular vesicles of size 0.5–1 μm, or big clusters of several specks of size 2–8 μm. Similar large-sized structures in autophagy have been described in only one other intestinal protozoan parasite, E. invadens, and their appearance coincided with the initiation of the encystation process (Piczarri et al., 2008). Since starvation is known to induce encystation in protozoan parasites such as Giardia lamblia and E. invadens (Avron et al., 1986; Lujan et al., 1996), it is possible that starvation triggered some Blastocystis cells to transit into granular or cyst forms, and that autophagy may promote the morphological changes through massive clusters of autophagic vacuoles. TEM is the most reliable method for monitoring autophagy (Mizushima, 2004). Highly polymorphic autophagic vacuoles were observed in amino-acid-starved and rapamycin-treated cells. Based on these randomly observed profiles, we postulate that the process of autophagy in Blastocystis starts from cytoplasmic membrane expansion to enclose organelles and other materials, and the formed autophagic vacuoles with double membranes are deposited into the central vacuole via invagination, so the resulting autophagic vacuoles exhibit three layers of membranes. The other scenario is the direct transfer of cytoplasmic components through vesicle invagination or tubular invagination, and subsequent budding of vesicles into the central vacuole, leading to the formation of autophagic vacuoles with one layer of membrane. Thus, the outer membrane of all the autophagic vacuoles found in the central vacuole is likely to be formed from the membrane of central vacuole via the invagination process. These single-membrane-bound structures eventually fuse with invaginated lysosomes. Because TEM images represent very thin sections of the sample, there is a possibility that the autophagic vacuoles observed in the central vacuoles are connected to the peripheral cytoplasm. Regardless of the origin, most autophagic vacuoles appeared to be accumulated in the central vacuole. The central vacuole is the largest organelle in Blastocystis vacuolar forms, and takes up approximately 90 % of the volume of the cell. It has been speculated to be an important organelle of this organism; however, the function of central vacuole is largely unknown (Tan, 2008). Through amino acid starvation and rapamycin treatment, the current study has suggested a role of the central vacuole in autophagy. Other studies using biochemical staining for carbohydrates or lipids have shown that some cells accumulate these two substances in the central vacuole, while some cells do not show any positive reactions to the stainings (Yoshikawa et al., 1995a, b). It is likely that the lipids and carbohydrates are actually released from degraded autophagic vacuoles, as autophagy serves to maintain homeostasis in healthy cells. The current study could also shed light on numerous unexplained observations with the central vacuole in earlier studies. Cytoplasmic inclusions within the central vacuole have been reported by others (Dunn et al., 1989; Pakandl, 1999), and they may be due to baseline autophagy in the parasites. The cytoplasmic projections into the central vacuole have been shown to increase in number during unfavourable culture conditions, such as in the presence of high concentrations of antibiotics (Boreham & Stenzel, 1993). It is possible that the cells upregulated autophagy in response to environmental stress.

It has been reported that mAb-1D5-treated Blastocystis pre-exposed to zVAD.fmk and/or cyclosporin A is not rescued from cell death (Nasirudeen & Tan, 2004, 2005). In the presence of either inhibitor, there seemed to be a compensatory mechanism, as the cells were able to trigger the mitochondrial-dependent death pathway in the absence of caspase-like activity, and vice versa. However, even though both caspase-like activity and MOMP were inhibited, and DNA fragmentation was abolished completely, the cells continued to die. Therefore, besides apoptosis, other cell death pathways might exist in Blastocystis, and be triggered upon mAb 1D5 induction. MDC-labelled vesicles were observed in mAb-1D5-treated Blastocystis, and 18.9 % of cells showed MDC-positive staining, suggesting that autophagy was triggered by mAb 1D5. The interplay between apoptosis and autophagy is complex, and not fully understood. Similar stimuli can induce either apoptosis or autophagy concomitantly,
sequentially, or in a mutually exclusive manner (Maiuri et al., 2007). It appeared that mAb 1D5 could trigger both apoptosis and autophagy, although apoptosis was still the major mode of cell death. However, when Blastocystis was pre-exposed to zVAD.fmk before incubation with mAb 1D5, the percentage of MDC-positive cells increased to 37.9%, indicating an upregulation of autophagy when caspase-like activity was blocked. This is consistent with findings in many metazoan cell lines that were sensitized to autophagic cell death in the absence of caspase activation (Vandenabeele et al., 2006; Yu et al., 2004). The current study also demonstrated the importance of MOMP in mAb-1D5-initiated autophagy, as when MOMP was inhibited by cyclosporin A, MDC-positive staining was abolished in mAb-1D5-treated cells, even with pre-treatment of zVAD.fmk. Mitochondrial damage has been reported to be a signal for autophagy in mammalian cells. Overexpression of mitochondrial calpain 10 has been found to cause mitochondrial swelling and increased autophagy, which was blocked by cyclosporin A (Arrington et al., 2006). It is likely that autophagy was triggered in Blastocystis via mAb-1D5-mediated mitochondrial dysfunction.

In this study, autophagy was found to be involved in the response to nutritional stress, and in cytotoxic-antibody-mediated cell death. Triggering autophagy in response to nutrient scarcity may represent an adaptive response by generating recycled metabolic substrates to maintain energy homeostasis (Meijer & Codogno, 2004; Tsukada & Ohsumi, 1993). However, when the nutrient supply via autophagy becomes ultimately depleted in prolonged starvation, massive autophagy may contribute to cell death (Eskelinen, 2005; Galluzzi et al., 2008). Autophagy may also serve as an alternative pathway for cells to die when they are stressed, such as with exposure to the cytotoxic antibody mAb 1D5, and it may become the main pathway of cell death when classical apoptosis is inhibited. The cellular target of mAb 1D5 has been recently identified to be a cell surface legumain (Wu et al., 2010), which is an asparagine endopeptidase that has been suggested to participate in helminth alimentary digestion of host proteins (Oliver et al., 2006). We postulate that inhibition of legumain activity by mAb 1D5 hinders the nutritional uptake of Blastocystis, and thus elicits a starvation response in the parasite.

In summary, we report the occurrence of autophagy in Blastocystis in response to various cellular stresses, and that the central vacuole of this organism plays an important role as a repository of autophagic vacuoles in the autophagy process.

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Fig. 7. Exposure to mAb 1D5 increased MDC-positive staining in Blastocystis. Cells were pretreated with 50 μM zVAD.fmk and/or 10 μM cyclosporin A (CA) for 30 min, and then incubated with mAb 1D5 or a non-specific IgM, anaerobically for 24 h at 37 °C. 3-MA treatment was performed by incubating cells with 20 mM 3-MA for 3 h prior to exposure to mAb 1D5. Following the different treatments, cells were stained with 0.05 mM MDC, and examined under a fluorescence microscope. (a) Fluorescent images of MDC staining of healthy and treated Blastocystis cells; bar 10 μm. (b) MDC staining of healthy and mAb-1D5-treated Blastocystis cells in the presence of 3-MA; bar 10 μm. (c) Bar chart comparing MDC-positive Blastocystis cells under different conditions. Five hundred cells were counted in each treatment. Values are means (±so) of two separate experiments, *P<0.05.


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