Ultrastructural analysis of the sporozoite of Cryptosporidium parvum

Laurence Tetley,1 Samantha M. A. Brown,1 Vincent McDonald2 and Graham H. Coombs1

Author for correspondence: Graham H. Coombs. Tel: +44 141 330 4777. Fax: +44 141 330 3516. E-mail: g.coombs@bio.gla.ac.uk

Cryopreparation of live sporozoites and oocysts of the apicomplexan parasite Cryptosporidium parvum, followed by transmission electron microscopy, was undertaken to show the 3D arrangement of organelles, their number and distribution. Profiles of parasites obtained from energy-filtering transmission electron microscopy of serial sections provided 3D reconstructions from which morphometric data and stereo images were derived. The results suggest that sporozoites have a single rhoptry containing an organized lamellar body, no mitochondria or conventional Golgi apparatus, and one or two crystalline bodies. Micronemes were shown to be spherical, numerous and apically located, and to account for 0.8% of the total cell volume. Dense granules were less numerous, larger, accounted for 5.8% of the cell volume, and were located more posteriorly than micronemes. A structure juxtaposed to the nucleus with similarities to the plastid-like organelle reported for other members of the Apicomplexa was observed. The detailed analysis illustrates the advantages of cryopreparation in retaining ultrastructural fidelity of labile or difficult to preserve structures such as the sporozoite of Cryptosporidium.

Keywords: Cryptosporidium sporozoite, ultrastructure, plastid, micronemes, rhoptry

INTRODUCTION

The protozoon Cryptosporidium parvum is a member of the phylum Apicomplexa and is included in its largest group, the coccidia (Tenter & Johnson, 1997). C. parvum is an obligately intracellular parasite which is responsible for disease in domestic livestock and humans. Infection occurs after ingestion of infective oocysts, from which the invasive sporozoite stage of the parasite emerges to enter cells lining the small intestine. Here the parasite proliferates, often resulting in diarrhoeal disease. Cryptosporidiosis is an important cause of death amongst infants in many tropical countries and, worldwide, is a potentially life-threatening complication in immunocompromised individuals (Fayer & Ungar, 1986; Goodgame, 1996).

Invasion of a host cell by coccidian sporozoites is a dynamic event of considerable interest as the attachment and entry processes involve the sequential secretion of the contents of discrete compartments from within the sporozoite. The released materials are thought to participate in a number of ways, including the penetration event itself and the formation of the vacuolar membrane which initially surrounds the intracellular parasite (see Coombs et al., 1997 for reviews). The machinery mediating this invasion process is collectively housed in the anterior region of the sporozoite and is known as the apical complex. Another unusual feature reported for C. parvum is the apparent lack of a mitochondrion (Current, 1989). There have been a number of detailed investigations, including analysis using serial sections, of the ultrastructure of sporozoites of Cryptosporidium and other coccidia, and the organelles they contain (Scholtyseck, 1979; Vivier, 1979; Uni et al., 1987; Tzipori, 1988; Current, 1989; Tzipori & Griffiths, 1998), but to date a full quantitative analysis and 3D reconstruction of the detailed ultrastructure of a coccidian sporozoite has not been published. One factor responsible for this deficiency has been the technical problem of analysing multiple serial thin sections for 3D reconstructions. In addition, difficulties have been encountered with the preservation of the integrity of the cells using conventional chemical fixation approaches. These problems have now been overcome by using...
cryotechniques and energy-filtering transmission electron microscopy (EFTEM) to image thick sections of the parasite. This approach has generated unique images of the parasites and in so doing has provided new insights into the subcellular organization of the sporozoite of *C. parvum*.

**METHODS**

**Parasites.** Oocysts of *C. parvum* were obtained from mice as described previously (Brown *et al.*, 1996). Oocysts were washed in RPMI and then either processed whole or excysted at 37 °C and the liberated sporozoites were harvested as described previously (Brown *et al.*, 1996). The oocysts or sporozoites were suspended to 10⁶ ml⁻¹ in RPMI medium for cryofixation.

**Cryopreparation.** Live parasites were impact frozen on a copper mirror using a Leica MM80 cryofixation system and then held at below -190 °C under liquid nitrogen prior to transfer to a Dewar-based cryosubstitution system, where specimens were slowly dehydrated in 1% osmium tetroxide in acetone for 6 d at -85 °C. After controlled warming (5 °C h⁻¹) to 0 °C and removal of the cryosubstitution medium by exchange with fresh acetone, the retrieved specimens were infiltrated with increasing concentrations of Spurr resin in acetone and finally polymerized in pure resin at 60 °C for 24 h.

**EFTEM and 3D reconstruction.** Sections (0.5 µm or 0.15 µm thick) were collected in ribbons of 12 on carbon-reinforced, Formvar-coated slot grids and stained in methanolic uranyl acetate for 15 min and then lead citrate for 10 min to ensure adequate contrast throughout the entire specimen thickness. Specimen contrast was enhanced by EFTEM imaging. Image series were photographically recorded at 80 kV by Zeiss 902 EFTEM at 100 eV energy loss to optimize contrast for 0.5 µm sections and at 30 eV for 0.15 µm sections. Colour-coded acetate traces performed on prints were used to derive digitized reconstruction profile data which were then input via a bit pad using '3D-HVEM' PC software (University of Boulder, CO, USA). Reconstructions were photographed from the monitor screen with an SLR camera using Fuji 400 daylight reversal film.

**RESULTS**

The distribution of most of the organelles in a sporozoite is depicted in the longitudinal section presented in Fig. 1. The section shows the characteristic conical-shaped, apical region (containing most of the organelles of the apical complex) of this invasive stage of the parasite and the posteriorly located nucleus. The various elements of the secretory machinery, notably the small spherical micronemes and larger dense granules, were present mainly in the anterior half of the cell. One or two large crystallloid bodies characteristically occurred close to the nucleus. There were two in the sporozoite presented: one anterior and one posterior to the nucleus. The crystallloid bodies contained a distinct periodic structure with a spacing of 35 nm. The nucleus itself exhibited peripheral, condensed chromatin with a central nucleolus and had distinct widely spaced nuclear membranes, the outer of which was studded with ribosomes. Close to the region anterior to the nucleus was observed an organelle of uncertain identity, which may be the equivalent of the plastid-like structure recently discovered in many members of the Apicomplexa (Williamson *et al.*, 1994; McFadden *et al.*, 1996; Kohler *et al.*, 1997; Wilson, 1998). Neither conventional mito-
**Fig. 2.** Stereo pair of 0.5-μm-thick sections of the conoid region of a sporozoite. The fused image reveals the single rhoptry (r), its tubular neck coursing by numerous micronemes to terminate at the apical complex tip. The neck of the rhoptry arches over a possible micropore (arrowhead), an invaginated specialization of the cell membrane. Bar, 0.25 μm.

**Fig. 3.** Structure of the apical region of a sporozoite. (a–c) Serial sections (0.15 μm) through the apical region to show details of the arrangement of organelles. The sequence illustrates the numerous spherical micronemes funnelling into the extended conoid where the single rhoptry terminates; dense granules are excluded from this region. The paracrystalline substructure inside the rhoptry bulb region is visible (arrowed). Bar, 0.25 μm. (d) Higher magnification of a rhoptry bulb showing the detail of the paracrystalline structure. Bar, 0.1 μm.
L. TETLEY and OTHERS

chondria nor a Golgi apparatus were observed in cryoprocessed sporozoites.

The whole apical region of C. parvum sporozoites was stereo-imaged using 0.5-μm-thick sections. The results (Fig. 2) revealed multiple micronemes but just a single rhoptry. This procedure also revealed a possible micropore structure which appeared to connect with the surface membrane just below the conoid (Fig. 2). However, this structure was not found in all of the sporozoites examined. Serial 0.15 μm sections through the same region as depicted in Fig. 2 confirmed that just a single rhoptry was apparent and showed details of the conoid, polar rings and the distribution of micronemes (Fig. 3a–c). The typically spherical dense granules were absent from this part of the sporozoite. The flask-shaped rhoptry consisted of a bulb-like structure attached to the thin membrane at the tip of the conoid by a long tubular neck. A conspicuously differentiated region in the centre of the bulb was also observed (Fig. 3a, arrow). It had a paracrystalline appearance, as shown in Fig. 3(d).

Three structures were routinely found associated with the nuclear region. On the anterior aspect of the nucleus, the outer nuclear membrane extended into the cytoplasm. Favourable sections showed this structure to contain nuclear pores or similar membrane fenestrations (Fig. 4a). A second structure was regularly found just anterior to the nucleus (Fig. 4b). The putative surrounding membranes were not well visualized with the protocol used, but the size and overall appearance of the organelle suggested that it may be the plastid-like structure which is now thought to be a common feature of all members of the Apicomplexa (McFadden et al., 1996; Hackstein et al., 1998). Separate from the nucleus, but always closely juxtaposed to it, was a ribosome-studded, 250-nm-diameter spherical organelle (Fig. 4c). This structure, which was usually observed at the posterior of the nucleus, but occasionally located anteriorly, was also present in encysted parasites (not shown).

Analysis of serial sections through an entire sporozoite (that presented in Fig. 3a) showed the relative shapes, sizes and distribution of each of the type of organelles and provided a means of estimating the contribution each made to the total volume of the sporozoite. The micronemes, dense granules and nucleus were observed to be essentially spherical, and their volumes were calculated on this basis. From the reconstruction of one sporozoite, the 167 micronemes present were shown to occupy 0.8% of the total cell volume (3.8 μm³), whereas the 18 dense granules accounted for 5.8% and the nucleus made up 16.8%. Analysis of the serial sections also revealed that there was no indication of a structure with morphological characteristics of a mitochondrion or a Golgi apparatus.

The organization of the organelles, which was revealed via serially sectioning whole sporozoites, is illustrated by the reconstructions prepared for stereo-viewing. The reconstruction from one eight-section series (partly shown in Fig. 3a–c) is presented in Fig. 5(a). This clearly shows that the micronemes are localized at the apical end of the sporozoite and that, in general, these organelles are more apical than are the dense granules. Similarly, the organization of nascent sporozoites within an oocyst is shown stereoscopically in Fig. 5(b). In this case, the considerable volume occupied by the oocyst's residual body and the outlines of individual sporozoites have been omitted to simplify visualization of the reconstruction. The nascent sporozoites, which were aligned within the oocyst with their apical ends towards the region containing the suture (not shown) through which the parasites emerge, were very similar in overall organization to the sporozoites after excystation.
DISCUSSION

Successful analysis of a cell's ultrastructure depends upon effective preparation of the material with cellular constituents stabilized near to their natural state. Chemical fixation can result in artefacts (Lee et al., 1982; Kellenberger et al., 1992), and retention of structural integrity in labile invasive stages such as sporozoites can also be poor with this method as membranes remain osmotically active after glutaraldehyde fixation (Bowers & Maser, 1988). Cryofixation overcome many of these potential problems and for this reason this approach was employed in this study. Alterations to cellular structure due to the formation of large ice crystals can be a problem with this procedure, but these are readily identified and so sections containing them can be avoided.

The sporozoite stage of C. parvum is both motile and short lived (certainly in vitro), its function being to invade host cells after it has emerged from the oocyst. This is achieved after attachment of the sporozoite's anterior end to the surface membrane of a prospective host cell and the secretion of materials from organelles of the apical complex. This study has revealed more detail on this complex in C. parvum sporozoites.

Conoid region and micropore

The sporozoite's conoid region, just below the tip of the polar ringed region (see Fig. 3a), is said to extend as the entry process proceeds (Scholtyseck et al., 1970) with the release of the contents of the secretory structures. A possible micropore, considered to be a feeding organelle (Chobotar & Scholtyseck, 1982), was observed below the conoid in the stereo pair images (Fig. 2) but was not detectable in all cells suggesting that, at most, it plays only a subsidiary role in the sporozoite's existence.

Rhoptry

The invasive stages of various apicomplexan parasites have been reported to contain between two and 20 rhoptries (Chobotar & Scholtyseck, 1982). Merozoites of C. parvum are thought to contain two of these secretory structures (Current, 1989), but the current study has revealed that sporozoites appear to possess only one. This suggests that the sporozoites of C. parvum may be unable to enter more than one host cell, which would contrast to the multiple invasions which sporozoites with many rhoptries, such as those of Toxoplasma, can apparently undertake.

Micronemes

The sporozoites examined during this study all contained a large number of micronemes (the 167 counted in the one reconstructed sporozoite appeared typical): this was not evident in the few published micrographs of sporozoites of Cryptosporidium species (Uni et al., 1987; Lumb et al., 1988). The name microneme means 'thread-like structure' and derives from earlier investigations of the invasive stages of other apicomplexan parasites in which the organelles appeared elongate (see Scholtyseck, 1979). However, in the current study the micronemes were apparently spherical, constant-sized
and relatively regularly spaced within the cytoplasm, with many situated at the tip of the apical complex alongside the rhoptry neck. These observations suggest that the elongate appearance of micronemes in conventionally fixed preparations may be artefactual. Support for this suggestion is provided by the observation that when micronemes purified from *Eimeria* sporozoites were exposed to glutaraldehyde they assumed an elongate shape similar to that observed in micrographs (Kawazoe *et al.*, 1992).

**Dense granules**

The population of spherical, 300-nm-diameter granules in the central region of the sporozoite were darkly stained and not morphologically distinguishable, even though subpopulations have been defined using immunoelectron microscopy (Bonnin *et al.*, 1995).

**Plastid-like body**

Several apicomplexan parasites have been reported to possess a single plastid-like structure (McFadden *et al.*, 1996). Unambiguous identification of this structure is not easy and the majority of current evidence depends upon the presence of multiple surrounding membranes (Kohler *et al.*, 1997; Hackstein *et al.*, 1998). In only a few cases has the presence of DNA been confirmed (McFadden *et al.*, 1996; Fichera & Roos, 1997; Wilson, 1998). The poor visualization of membranes that is achieved using cryopreservation means that this study allows only tentative conclusions concerning the presence of a plastid-like structure in *C. parvum*. However, the size and position of the structure that appeared as a ribosome-filled spheroid surrounded by a double membrane-sized halo just anterior to the nucleus (Fig. 4) fits the description of the plastid-like bodies in other apicomplexan parasites. Nevertheless, it must be remembered that *C. parvum* differs from other coccidia in a number of ways and confirmation that the parasite indeed has a plastid-like body, and its location, will require some specific in situ labelling, as has been carried out with *Toxoplasma* (McFadden *et al.*, 1996).

**Mitochondrion**

One major aim of this study was to determine whether *C. parvum* indeed lacks a mitochondrion. Such a structure has been reported for all other coccidia investigated, including, surprisingly, the merozoites of *Cryptosporidium muris* (Uni *et al.*, 1987). However, this study has confirmed the apparent absence from *C. parvum* sporozoites of any structure similar morphologically to mitochondria found in other apicomplexan parasites. This correlates well with the energy metabolism of the parasite and the reported absence of Krebs cycle enzymes (Denton *et al.*, 1996; Coombs *et al.*, 1997; Entraña & Mascaro, 1997) as well as its lack of sensitivity to respiratory inhibitors (Brown *et al.*, 1996). Nevertheless, it remains a surprise that such apparently closely related species (for instance *C. parvum* and *C. muris*) should apparently differ so fundamentally. We are investigating the ultrastructure of *C. muris* to clarify this position.

**ACKNOWLEDGEMENTS**

S. M. A. B. was supported by a BBSRC CASE studentship with Pfizer Animal Health Discovery.

**REFERENCES**


Received 26 May 1998; revised 29 July 1998; accepted 25 August 1998.