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Ultrastructure of the *Babesia divergens* free merozoite

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ABSTRACT

The invasive form of the apicomplexan parasite *Babesia divergens*, the free merozoite, invades the erythrocytes of host vertebrates, leading to significant pathology. Although invasion is an active process critical for parasite survival, it is not yet entirely understood. Using techniques to isolate the viable free merozoite, as well as electron microscopy, we undertook a detailed morphological study and explored the sub-cellular structure of the invasive *B. divergens* free merozoite after it had left the host cell. We examined characteristic apicomplexan features such as the apicoplast, the inner and discontinuous double membrane complex, and the apical complex; some aspects of erythrocyte entry by *B. divergens* were also defined by electron microscopy. This study adds to our understanding of *B. divergens* free merozoites and their invasion of human erythrocytes.

1. Introduction

Apicomplexa is a large phylum of parasites that greatly affect humans and animals. These include *Plasmodium*, *Toxoplasma*, *Theileria*, *Eimeria*, *Babesia*, and *Cryptosporidium* (Cowman and Crabb, 2006). *Babesia divergens* is an obligate hemoparasite transmitted by the *Ixodes ricinus* tick. The parasite is pathogenic within a vertebrate host. The primary host consists of cattle, which are afflicted with redwater fever (Zintl et al., 2003); *B. divergens* also affects reindeer (Wiegmann et al., 2015) and occasionally, humans (Hildebrandt et al., 2013). In humans, infection of red blood cells (RBC) can lead to malaria-like febrile illness (Lobo et al., 2012). Patients, who are immunocompromised, usually as a result of splenectomy or spleen dysfunction, suffer acute infections (Vannier et al., 2015). *B. divergens* is also a potential threat to the blood supply (Castro et al., 2014; Cursino-Santos et al., 2014).

Once the host is bitten by an infected tick, *B. divergens* sporozoites directly invade host erythrocytes, after which the parasite asexual cycle commences, followed by multiplication by binary fission. Seven distinct stages are maintained within RBC, that is, single rings, paired figures, double rings, double paired figures, tetrad or Maltese Cross, quadruple rings, and multiple intraerythrocytic parasites. Double or more unattached parasite cells can egress at any time. The only parasite form that is found outside the cell is the invasive merozoite that has been released, after which the free merozoite invades a new RBC (Cursino-Santos et al., 2015). The appearance of host symptoms is caused by the continuous invasion and destruction of erythrocytes by *B. divergens*, a critical process not yet entirely understood. Optical and electron microscopy assays indicate that *B. divergens* invades RBCs rapidly, probably within 45 (Sun et al., 2011) to 60 s (Montero et al., 2009). The free merozoite recognizes the RBC and establishes contact with them before being internalized through the formation of a parasitophorous vacuole (PV) by invagination of the erythrocyte membrane. *B. divergens* quickly dissociates with the PV to make direct contact with the RBC cytoplasm (Repnik et al., 2015).

The ultrastructure of *B. divergens* free merozoites is not well characterized, in contrast to the intraerythrocytic stages of *B. divergens* (Baumeister et al., 2015; Gorenflot et al., 1991; Friedhoff and Scholtyseck, 1977; Repnik et al., 2015) or free merozoites of *Babesia microti* (Rudzinska et al., 1976), *Toxoplasma gondii* (Aikawa et al.,

Abbreviations: RBC, red blood cell; TEM, transmission electron microscopy; IMC, inner membrane complex; PV, parasitophorous vacuole; RT, room temperature; ER, endoplasmic reticulum.

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1977) and *Plasmodium falciparum* (Aikawa et al., 1978; Bannister et al., 2000; Hanssen et al., 2013), among other apicomplexan parasites. Here we analyzed the free merozoite of *B. divergens* using transmission electron microscopy (TEM) and found several well-conserved structures, as well as some architectural features of the invasion process.

2. Materials and methods

2.1. Ethics statement

Human A+ blood from healthy volunteer donors was used to maintain *B. divergens* blood stage cultures. The blood and protocol were approved for use by the Blood Transfusion Center, Madrid, Spain. Donors provided informed written consent for use of their blood for research purposes.

2.2. Parasite propagation

Babesia divergens cultures (Bd Rouen 1987 strain) were maintained *in vitro* in human A+ RBC at 5% hematocrit (Gorenflot et al., 1991). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Free merozoite isolation

Cultures were grown to ~20% parasitemia and centrifuged (600*g*, 5 min). The resulting supernatant was filtered through 5 μm and 1.2 μm Versapor syringe filters (Pall Corporation, Ann Arbor, MI). Merozoites were then pelleted (2000*g*, 5 min). Free merozoites were fixed immediately for electron microscopy.

2.4. Electron microscopy

For ultrastructural analysis, *B. divergens in vitro* cultures and freshly isolated *B. divergens* free merozoites were fixed in 0.1 M Na_2HPO_4 pH 7.4, 2% glutaraldehyde and 4% paraformaldehyde (2 h, room temperature (RT)). Samples were washed three times in Na_2HPO_4 buffer at 4 °C and post-fixed with a mixture of 1% osmium tetroxide and 1% potassium ferricyanide (1 h, 4 °C), 0.15% tannic acid (1 min, RT) and 2% uranyl acetate (1 h, RT). Samples were dehydrated in increasing concentrations of ethanol (50, 75, 90, 95, 100%; 10 min each, 4 °C) and infiltrated in epoxy-resin at RT using increasing concentrations of resin (25, 50, 75, 100%) that was polymerized at 60 °C for 48 h. Ultrathin sections (70–100 nm) were obtained with a RMC MT6000_XL ultramicrotome, harvested on Formvar-coated copper grids and stained with saturated uranyl acetate and 2% lead citrate following standard procedures. Sections were analyzed in a Tecnai 12 FEI microscope operated at 120 kV and images were registered in micrographs or on a CCD 1k Gatan camera.

3. Results and discussion

Transmission electron microscopy analysis of the ultrastructure of the free merozoite outside the RBC showed that the *B. divergens* merozoite shares some characteristics with free merozoites of other members of the phylum Apicomplexa. The *B. divergens* merozoites had a polarized morphology and a well-developed apical end (Fig. 1A). Double membrane organelles, apical organelles known to be involved in invasion of erythrocytes, the inner membrane complex (IMC) of the apicomplexan cell interior and the apicoplast were identified in *B. divergens* free merozoites (Fig. 1A–D).

3.1. The subcellular structure of the divergens free merozoite

Longitudinal sections of merozoites were approximately 3 μm in length and generally had an elongated oval shape. Double-membrane organelles, which are characteristic of eukaryotic cells, were embedded in the cytoplasm. In longitudinal sections, the nucleus was large, with a homogenous granular nucleoplasm. In most cases, the nucleus was found on one side of the merozoite (Fig. 1C), although the nucleus and the apical complex were occasionally observed at opposite ends of the parasite. Double-membrane oval structures were scattered within the merozoite cytoplasm, similar to the mitochondria seen in free merozoites of *B. microti* (Rudzinska et al., 1976) and *Babesia* sp. (Holman et al., 2005) and in the intraerythrocytic forms of *Babesia caballi* (Kawai et al., 1999) and *Babesia duncani* (Conrad et al., 2006) (Fig. 1). We also detected intracytoplasmic empty vacuoles surrounded by a single cytoplasmic membrane, similar to those described for *B. microti* free merozoites (Fig. 1C). These empty "pseudo-food vacuoles" denote the space previously occupied by the invaginated host erythrocyte cytoplasm in the intraerythrocytic stages (Rudzinska, 1976). Compatible with this observation, we detected large hemoglobin inclusions in the intraerythrocytic *B. divergens* stage (Terrón, unpublished data).

We also studied cell features specific to apicomplexan parasites. The *B. divergens* merozoites are delimited by a continuous, enveloping outer membrane. In some cases, a granular material covered the entire surface of the free merozoite (Fig. 1D, arrowheads). In electron microscopy, a similar granular material is also clearly visible in the surface coat of *B. microti* (Rudzinska et al., 1976), *Babesia. bovis* (Smith et al., 1981), and *Plasmodium* (Aikawa et al., 1978) free merozoites. The merozoite surface of apicomplexan parasites contains integral, invasion-associated membrane proteins (Lobo et al., 2012). In *B. divergens*, Bd37 is considered the major surface antigen and is implicated in RBC adhesion and internalization (Delbecq et al., 2008).

Our ultrastructural analyses demonstrated an apicoplast in the free merozoites of *B. divergens.* Apicoplasts are plastid organelles similar to plant chloroplasts, of interest because of their evolutionary and potential therapeutic implications (Waller and McFadden, 2005). The *B. divergens* apicoplast is a multi-membranous organelle with similar features as the intraerythrocytic stage apicoplasts of *B. bovis* (Caballero et al., 2012), *P. falciparum* (Lemgruber et al., 2013) and *T. gondii* (Parsons et al., 2007). The organelle was approximately 500 nm in diameter, clearly demarcated with four overlying membranes, and was generally located next to the IMC (Figs. 1C and 2A). This differs from the intraerythrocytic stage of *B. bovis* and *P. falciparum* (Hanssen et al., 2013; Lemgruber et al., 2013), in which the apicoplast is proximal to the nucleus.

The IMC has been observed in some intraerythrocytic forms of *B. divergens* (Baumeister et al., 2015; Friedhoff and Scholtyseck, 1977; Gorenflot et al., 1991; Repnik et al., 2015). Here, the IMC structure appeared to be well conserved in the *B. divergens* free merozoite interior. This complex has a cisternal structure formed by a double membrane that gives stability and shape to the merozoite. The IMC appeared to be very close to the membranes of the endoplasmic reticulum (ER) (Fig. 2A). In *P. falciparum*, the IMC is continuous with the ER and the nucleus, a finding consistent with the possibility that the IMC develops from a post-Golgi compartment (Agop-Nersesian et al., 2010; Hanssen et al., 2013). The IMC extended discontinuously over the interior surface of the *B. divergens* merozoite. Some discontinuities were seen at the midpoint of the merozoite (Figs. 1D and 2A and B). This uneven IMC distribution was previously observed in *P. falciparum* and is clearly involved in the organization of the motility apparatus (glideosome) of free merozoites. Gaps or openings in the IMC might be involved in the interruption of the adhesion and de-adhesion cycles that occur when the merozoite