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Tubular structures associated with *Babesia caballi* in equine erythrocytes in vitro

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Abstract In-vitro-propagated *Babesia caballi* parasites were examined by scanning and transmission electron microscopy. Many small pores were observed over the entire surface of infected erythrocytes on scanning electron microscopy, and on transmission electron microscopy these small pores were found to be openings of tubular structures. By the examination of a number of infected cells the tubular structures were found to be connected with the parasite, and this observation might indicate that the tubular structures arose from the edge of the parasite and terminated at an invagination on the surface of the erythrocyte. These findings suggest that intraerythrocytic stages of *B. caballi* come into direct contact with culture medium.

Introduction

Babesia caballi and *B. equi*, members of the phylum Apicomplexa, are intraerythrocytic protozoa that cause piroplasmosis in the horse, donkey, mule, and zebra (Purnell 1981). Piroplasmosis is characterized by fever, anemia, anorexia, and, during the later stages of the disease, icterus and hemoglobinuria. Equine piroplasmosis is endemic in tropical and subtropical regions of Central America, South America, Africa, Asia, and southern Europe (Knowles 1988). Due to the international horse trade there is a constant risk for the introduction of *Babesia* carrier animals into nonendemic areas such as North America, Australia, and Japan (Böse and Hentrich 1994; Knowles 1996).

As stated by Levine (1971), the genus *Babesia* can be divided into two groups: large types (more than 3 μm long) and small types (less than 3 μm long). According to Riek (1968), merozoites of *B. caballi* occur in erythrocytes as pyriform bodies measuring 2.15–4.0 μm in length and about 2.0 μm in width and as spherical or ameboid bodies measuring 1.5–3.0 μm in diameter. The pyriform parasites frequently occur in pairs at acute angles to each other. Therefore, *B. caballi* can be classified as a large-type *Babesia*, like *B. bigemina* (Purnell 1981).

In contrast to *B. caballi*, *B. equi*, whose merozoites measure about 2.0 μm in length and about 1.0 μm in width, belongs to the small-type *Babesia* group (Mahoney et al. 1977). Holbrook et al. (1968) have described the intraerythrocytic reproduction of *B. equi*, in which a ''Maltese-cross arrangement'' of merozoites occurs. This arrangement does not occur in the case of *B. caballi*. Thus, *B. caballi* and *B. equi* differ in size, shape, and development and can therefore easily be distinguished from one another in cases of double infection (Holbrook et al. 1968).

Recently, an in vitro *B. caballi* culture system for antigen production was developed (Bhushan et al. 1991; Avarzed et al. 1997). Relatively higher levels of parasitemia can be achieved in this in vitro system as compared with animal infection. Furthermore, the immune system affects the observed stages of parasites, whereas in the in vitro system, effects of the immune system are avoided. The rapid proliferation of in-vitro-cultured *Babesia* parasites enables the study not only of the fine structure of the parasite but also of its modes of replication (Droleskey et al. 1993).

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Development of the intraerythrocytic stage of *B. caballi* has been documented by light microscopy, and primary reports on the fine structure of the intraerythrocytic stages of *B. caballi* have been published (Simpson et al. 1963, 1967; Holbrook et al. 1968). These reports include illustrations of the morphological features of merozoites, trophozoites, and dividing parasites. However, detailed descriptions of the ultrastructural changes in *B. caballi*-infected erythrocytes remain incomplete. Using scanning and transmission electron microscopy, the present study elucidated some morphological characteristics of equine erythrocytes infected with *B. caballi* and revealed the development of tubular structures in vitro.

Materials and methods

In vitro cultivation of *Babesia caballi*

The USDA strain of *B. caballi* was grown in vitro by the method of Avarzed et al. (1997). The parasite was cultured in equine erythrocytes [10% (v/v) hematocrit] in buffered RPMI 1640 medium (Dainippon Pharmaceutical Co., Ltd., Osaka) supplemented with 40% equine serum, L-glutamine, and antibiotics. Cultures were incubated at 37 °C in an atmosphere of 5% CO_2 in humidified air. Development of the parasite in vitro was monitored by microscope observation of Giemsa-stained thin smears until the level of parasitemia reached about 12%.

Electron microscopy procedures

For scanning electron microscopy, blood specimens were fixed for 2 h in 2.5% (v/v) glutaraldehyde buffered with 0.1 *M* phosphate buffer (pH 7.4) at 4 °C. They were postfixed in 1% (w/v) osmium tetroxide for 1 h and then spread onto poly-L-lysine-coated coverslips (Matsunami Glass, Japan). They were then dehydrated in a graded ethanol series and dried in a JEOL JFD-300 freeze-drier (JEOL, Tokyo, Japan). Specimens were mounted on aluminum stubs, sputter-coated with gold, and observed and micrographed in a Jeol SC-630 scanning electron microscope.

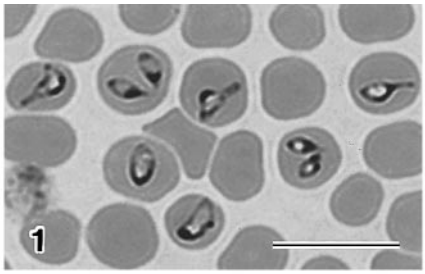
Specimens for transmission electron microscopy were fixed for 2 h in 2.5% (v/v) glutaraldehyde buffered with 0.1 *M* phosphate buffer (pH 7.4) at 4 °C They were postfixed in 1% (w/v) osmium tetroxide for 1 h, then dehydrated in a graded series of alcohols, treated with propylene oxide for 15 min and were then embedded in Epon 812. The blocks obtained were cut with an ultramicrotome (Porter-Blum MT-2; Ivan Sorvall, Inc.. Norwal, Conn., USA) equipped with a diamond knife (Diatome, USA). The sections were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM-200X transmission electron microscope.

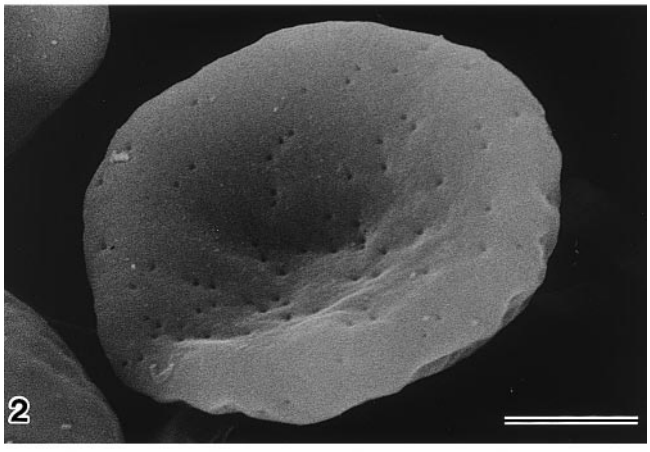
Results

Giemsa-stained thin smears of cultured parasites examined light microscopically revealed various intraerythrocytic stages such as single or paired pyriforms and single spherical or ameboid forms (Fig. 1). No change was seen on infected erythrocytes such as the dots and clefts observed on erythrocytes infected with *Plasmodium*.

Scanning electron microscopy revealed slight indentations and many small pores evenly distributed over the entire infected erythrocyte surface (Fig. 2). Each pore was round or oval-shaped, measuring approximately 40 nm in diameter.

On transmission electron microscopy, paired pyriform parasites, ameboid forms, and dividing forms of *Babesia caballi* were subcentrally located in host erythrocytes. In several infected erythrocytes, some vesicles were seen in the host cytoplasm (Fig. 3). The vesicles





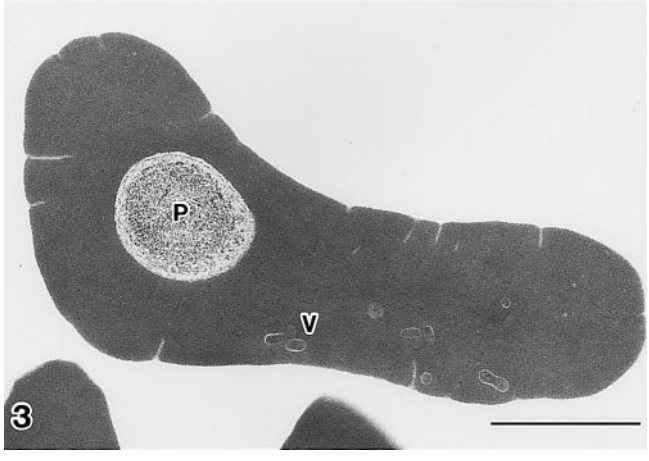


Fig. 1 Light micrograph of a Giemsa-stained thin smear of cultured parasites, showing various intraerythrocytic stages. Magnification X 2 400. *Bar* 10 μm

Fig. 2 Scanning electron micrograph of a *Babesia caballi*-infected equine erythrocyte. Magnification X 25 000. *Bar* 1 μm

Fig. 3 Transmission electron micrograph of a *B. caballi*-infected equine erythrocyte with many tubular structures (*P* Piroplasm, *V* vesicles). Magnification X 28 000. *Bar* 1 μm

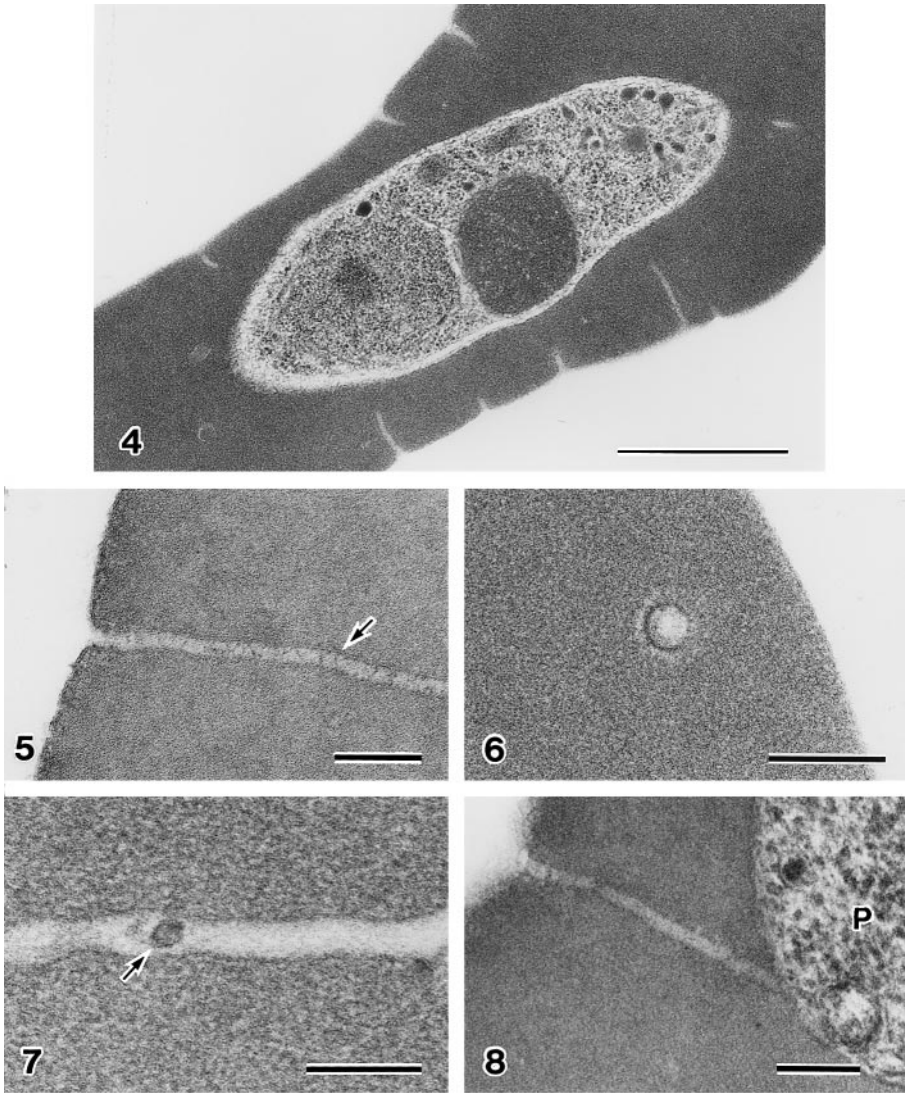


Fig. 4 Transmission electron micrograph of a *B. caballi*-infected equine erythrocyte, showing many tubular structures along the extreme edge of the host cell cytoplasm. Magnification X 30 000. *Bar* 0.5 μm
Fig. 5 High-magnification electron micrograph of a longitudinal section of a tubular structure. A unit membrane is clearly recognizable around the tubular structure (*arrow*). Magnification X 60 000. *Bar* 250 nm
Fig. 6 High-magnification electron micrograph of a transverse section of a tubular structure. Magnification X 84 000. *Bar* 250 nm
Fig. 7 High-magnification electron micrograph of a longitudinal section of a tubular structure. A particle is visible in the lumen of the tubular structure (*arrow*). Magnification X 85 000. *Bar* 250 nm
Fig. 8 Transmission electron micrograph of a *B. caballi*-infected equine erythrocyte (*P* Piroplasm). The tubular structures arise from the edge of the parasite and terminate at an invagination on the surface of the erythrocyte. Magnification X 55 000. *Bar* 250 nm

were surrounded by an area of low electron density measuring approximately 0.1 nm in width and were round or elongated. They were scattered throughout the host cytoplasm. The density of these vesicles was similar to that of the host cell cytoplasm (Fig. 3).

Many tubular structures were often seen in the infected cells (Fig. 3). Such structures were comparable with the small pores seen by scanning microscopy. When the structures were sectioned longitudinally, they were straight or slightly curved and were usually located along the extreme edge of the host cell cytoplasm (Fig. 4). These tubular structures had a common membrane with that of the host cell, and a unit membrane was clearly recognizable around the tubular structures (Fig. 5, arrow). On transverse sections the tubular structures were round and had a diameter of about 50 nm (Fig. 6). A unit membrane was observed around tubular structures, and the density of the central lumen was lower than that of the host cell cytoplasm (Fig. 6). Occasionally, some particles were found in the lumen of the tubular structure (Fig. 7, arrow). By the examination of a number of infected cells, tubular structures were found to be connected with the parasite (Fig. 8), and this observation might indicate that tubular structures arose from the edge of the parasite and terminated at an invagination on the surface of the erythrocyte.

Examination of nonparasitized equine erythrocytes by scanning and transmission electron microscopy revealed no surface indentation, tubular structure, or vesicle comparable with those observed in the parasitized cells.

Discussion

In this study we made ultrastructure observations of in-vitro-cultured *Babesia caballi* by scanning and transmission electron microscopy. Many small pores were observed for the first time over the entire surface of infected erythrocytes by scanning electron microscopy, and on transmission electron microscopy these small pores were found to be the openings of tubular

structures. Although Simpson et al. (1963, 1967) observed elongated microtubules in the cytoplasm of *B. caballi* parasites in specimens prepared from splenectomized ponies, they did not describe tubular structures in infected erythrocytes. The lack of tubular structures in Simpson et al.'s studies might be explained by the use of old methods of fixation specimen processing and the poor performance of electron microscopy about 30 years ago. Moreover, their observations were made using blood samples with low levels of parasitemia obtained from infected animals. In the present study, ultrastructure observations were made on cultured samples, including a great number of parasites in all stages of intraerythrocytic development. Previous studies have shown that the *B. divergens* and *B. bovis* fine structure in vitro is indistinguishable from that in vivo (Gorenflot et al. 1991; Holman et al. 1993). It is thus possible that the tubular structures associated with *B. caballi* infection were found in a higher percentage of parasitized cells prepared in vivo by the same fixation (and subsequent) methods used in the present study.

Changes on the surface of infected erythrocytes have been reported in *Babesia* and *Plasmodium* infections. Sun et al. (1983) observed perforations on the surface of erythrocytes infected with *B. microti*. Bodammer and Bahr (1973) observed deep invaginations or focal depressions on the surfaces of erythrocytes infected with *P. berghei* NYU-2; these structures were postulated to be "metabolic windows" permitting parasites access to metabolites outside the cell. However, these perforations and "metabolic windows" differ in size and number from the small pores observed in the present study on membranes of erythrocytes infected with *B. caballi*.

In the cytoplasm of *Babesia*-infected erythrocytes, some characteristic structures in addition to the parasites have been reported, including translucent single membrane-bound vesicles, aggregated tiny vesicles, tightly coiled membranes, and Maurer's clefts (Rudzinska 1981). The presence of Maurer's clefts has been reported in *B. bovis* trophozoites, especially in multiple infections of a single bovine erythrocyte (Todorovic et al. 1981). This structure has also been recognized in trophozoites of *B. bigemina*, *B. divergens*, *B. microti*, and some *Plasmodium* species (Saal 1964 ; Rudzinska 1981; Atkinson and Aikawa 1990).

According to Aikawa et al. (1986) and Atkinson and Aikawa (1990), cytoplasmic clefts associated with *P. falciparum* extended from the parasite and the surrounding parasitophorous vacuole membrane into the erythrocyte cytoplasm, and these authors suggested that they were a route of transport for parasite antigens through the erythrocyte cytoplasm to the erythrocyte membrane. However, in the present study, erythrocytes infected with *B. caballi* contained neither cytoplasmic clefts nor a parasitophorous vacuole membrane in the erythrocyte cytoplasm. These findings suggest that the tubular structures associated with *B. caballi* differ morphologically and functionally from the cytoplasmic clefts seen in erythrocytes infected with *P. falciparum* and other *Babesia* species.

The ultrastructure of *B. equi* has been described, a unique morphological feature of which is the presence of tubular food vacuoles that extend from intraerythrocytic parasites (Frerichs and Holbrook 1974). When the tubular food vacuoles of *B. equi* extended to the periphery of the erythrocyte, its central lumen was often in direct contact with plasma. Although the function of the tubular structures remains unknown, it has been speculated that they play a role in the uptake of nutrients from host plasma (Frerichs and Holbrook 1974).

In the present study we found that the tubular structures of *B. caballi*-infected erythrocytes extended from the surface of the cell membrane directly to the parasite, suggesting that intraerythrocytic stages of *B. caballi* come into direct contact with culture medium or host plasma. As described for *B. equi*, if the tubular structures associated with *B. caballi* are related to ingestion by parasites, our findings would suggest that the erythrocytic stages of *B. caballi* obtain nutrients directly from the area outside of erythrocytes. Further studies are needed to clarify the process of formation and biological function of the tubular structures.

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