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International Journal for Parasitology 34 (2004) 1221–1227
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Blocked stomodeal valve of the insect vector: similar mechanism of transmission in two trypanosomatid models

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Received 14 May 2004; received in revised form 20 July 2004; accepted 29 July 2004

Abstract

The regurgitation of metacyclic stages from the sand fly cardia is thought to be the prevailing mechanism of *Leishmania* transmission. This regurgitation may result through damage of the stomodeal valve and its mechanical block by the parasites. We found this phenomenon in three sand fly–*Leishmania* models and also in avian trypanosomes transmitted by *Culex* mosquitoes. *Phlebotomus duboscqi*, *Phlebotomus papatasi*, *Lutzomyia longipalpis*, and *Culex pipiens* were membrane-fed on blood containing *Leishmania major*, *Leishmania chagasi* (syn. *infantum*) and an unidentified avian *Trypanosoma* from *Trypanosoma corvi* clade, respectively. Females with the late-stage infections were processed for the optical and transmission electron microscopy. Localization of the parasites and changes to the stomodeal valve were in some aspects similar in all vector–parasite pairs studied: (i) a large plug of flagellates was observed in cardia region, (ii) parasites were attached to the chitin lining of the stomodeal valve by the formation of zonal hemidesmosome-like plaques. *Leishmania* promastigotes were found both attached to the valve as well as unattached in the lumen of midgut. The stomodeal valve of infected sand flies was opened, its chitin lining was destroyed and the unique filamentous structures on the apical end of cylindrical cells were degraded. In the *Culex*–*Trypanosoma* model, the whole population of epimastigotes was found in close contact with the chitin lining, and degenerative changes of the valve were less pronounced. We suggest that the phenomenon involving a blocked valve facilitating the regurgitation of parasites into the vertebrate host may occur generally in heteroxenous trypanosomatids transmitted by the bite of nematoceran Diptera.

Keywords: Stomodeal valve; Midgut; Sand fly; Mosquito; *Leishmania*; *Trypanosoma*

1. Introduction

Transmission from an insect vector to a vertebrate host is a key moment in the life cycle of heteroxenous parasites, including trypanosomatids of the genera *Leishmania* and *Trypanosoma*. The anatomy of the alimentary tract of the vector and the localization of the parasite development within the tract play important roles in this event. The insect alimentary canal is formed by three major regions: foregut, midgut and hindgut. The foregut and hindgut arise as an ectodermal invagination and retain the capacity to secrete a chitin layer continuous with the integumentary cuticle while the midgut is of endodermal origin and its surface is covered by microvilli. The junction between foregut and midgut is located at the point where the midgut epithelium is linked to the reflected wall of the oesophagus. In Diptera, this complex structure is called the cardia and it consists of the stomodeal valve (valvula cardiaca, cardiac valve) and the most anterior part of the midgut epithelium (for review see Romoser, 1996).

The stomodeal valve is of ectodermal origin and consists of a ring of cylindrical epithelial cells that bulges as mushroom-shaped extension of foregut cells into the cardia region. In nematoceran Diptera, including mosquitoes and sand flies, the main role of the stomodeal valve is to ensure the 'one-way' flow of food during feeding and to prevent regurgitation of the gut contents. In sand flies the valve is also supposed to play a role in controlling the destination of meals; during feeding the blood is directed to

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0020-7519/$30.00
doi:10.1016/j.ijpara.2004.07.010

the midgut while the sugar meal is diverted to the crop (Tang and Ward, 1998).

Sand fly females infected with *Leishmania* have difficulty in engorging the meal during the second blood-feeding and they probe several times when biting (for review see Molyneux and Jefferies, 1986). This phenomenon of the 'blocked fly', first mentioned by Shortt and Swaminath (1928), was later accepted by other authors. Parasites colonizing the anterior part of the gut, limit the flow of the bloodmeal, and cause a backflow that carries the parasites and results in their deposition into the host skin (Killick-Kendrick et al., 1977; Jefferies et al., 1986; Molyneux and Jefferies, 1986). Moreover, promastigotes in the cardia region are entrapped in a viscous gel-like plug that occludes the gut lumen and contributes to the blockage of the stomodeal valve (Lawyer et al., 1987, 1990; Walters et al., 1987, 1989a,b; Killick-Kendrick et al., 1988; Lang et al., 1991; Rogers et al., 2002). Recently, Stierhof et al. (1999) showed that the gel-like mass is formed mainly by a parasite-derived mucin-like filamentous proteophosphoglycan.

In addition to this mechanical block, *Leishmania* infections cause pathological changes in the sand fly alimentary canal. Schlein et al. (1991, 1992), working on *Phlebotomus papatasi* and *Leishmania major*, described damage to the chitin lining of the stomodeal valve by heavy infections, presumed to be caused by chitinolytic enzymes of the parasite. These pathological changes may influence the function of the valve and lead to the regurgitation of parasites with a backflow of ingested blood. Transmission occurs when infective parasites from the mass in the cardia region are regurgitated with repeated pump pulsation which are then ejected into the host tissue (Schlein et al., 1992; Schlein, 1993).

In the present work, we investigated the pathological changes of the stomodeal valve in different *Leishmania*-sand fly models and showed that the phenomenon of the 'blocked valve' occurs more generally in trypanosomatids transmitted by the bite of nematoceran Diptera. In addition, we described a filamentous structure in cylindrical cells of the stomodeal valve, which could be important for understanding the pathological changes that occur in infected vectors.

2. Materials and methods

2.1. Insect colonies and parasite cultures

Laboratory colonies of *Phlebotomus duboscqi* (origin from Senegal), *P. papatasi* (Turkey), *Lutzomyia longipalpis* (Jacobina, Brazil), and *Culex pipiens quinquefasciatus* (India) were used. Adults were maintained on a 50% sucrose diet, in >70% relative humidity at 25 °C. Females were allowed to feed on anaesthetized mice once a week. Different trypanosomatid parasites were used: *L. major* LV561 (MHOM/IL/67/LRC-L137 Jericho II), *Leishmania chagasi* (syn. *infantum*) M4192 (MHOM/BR/76/150406) and *Trypanosoma* sp. (ICUL/CZ/1999/CUL1), an avian trypanosome isolated from *Culex pipiens pipiens* (Votypka et al., 2002); based on phylogeny inferred from small-subunit rDNA this trypanosome belongs to *Trypanosoma corvi* clade (Votypka et al., 2004). Parasites were maintained on SNB-9 blood agar.

2.2. Experimental infections

Female sand flies, 4–6 days old, were infected by feeding through a chick-skin membrane on heat inactivated rabbit blood containing 10^6 *Leishmania* promastigotes/ml from a 5-day old culture. Three natural parasite–vector combinations were tested: *L. major–P. papatasi* and *P. duboscqi*, and *L. chagasi–L. longipalpis*.

Twelve to 14-day old *Culex* females were infected with *Trypanosoma* sp. using membrane feeding on duck blood containing 10^7 promastigotes/ml from a 7 day old culture. Laboratory infections mimic well the natural infections observed in wild-caught mosquitoes (Votypka, unpublished).

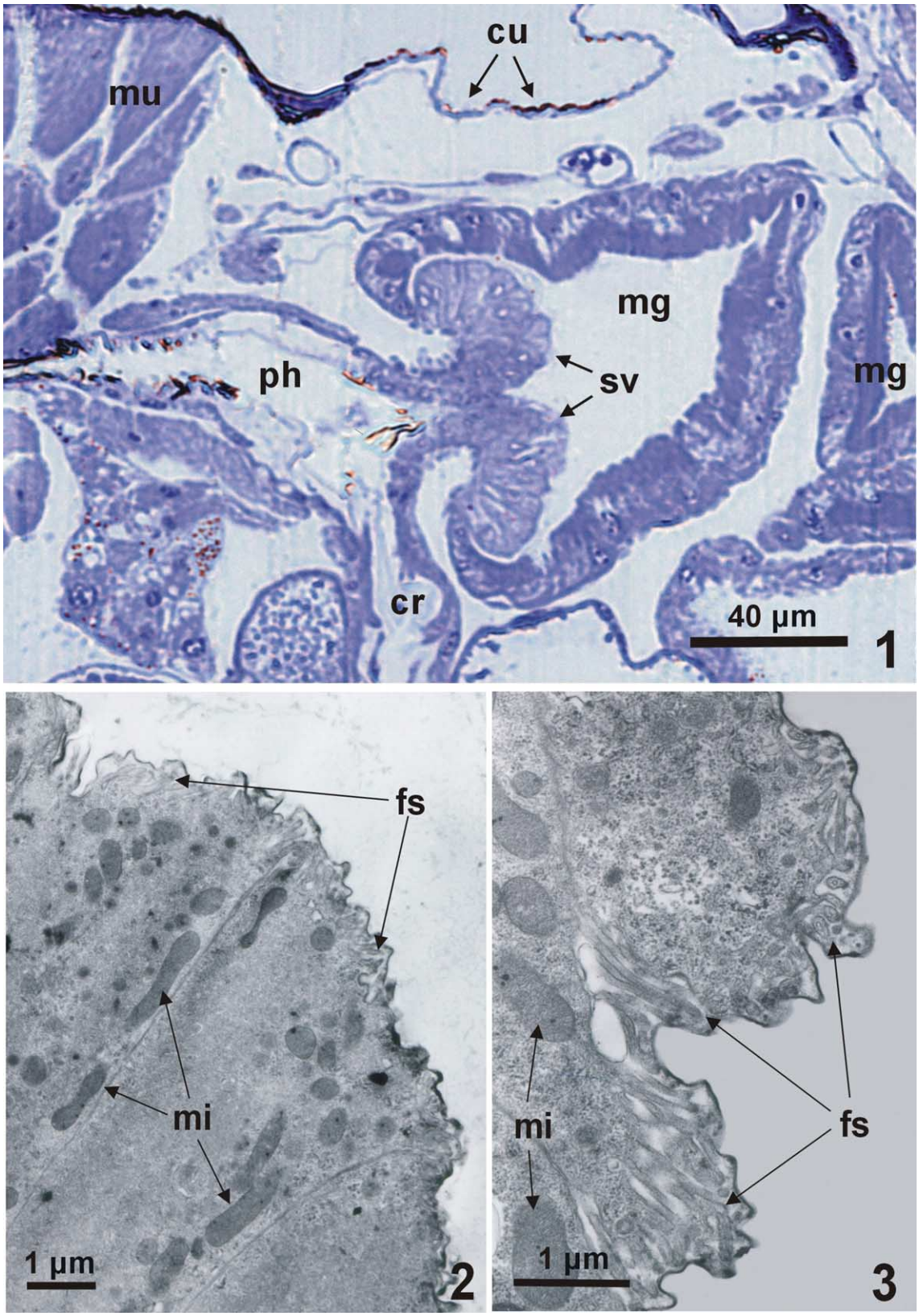
2.3. Light and transmission electron microscopy

Engorged female sand flies and mosquitoes were separated and maintained for 10–12 days at 23 °C and a 14 h light/10 h dark photoperiod. They had free access to 50% sucrose solution. Females were anaesthetized on ice and fixed in 4% glutaraldehyde in PBS for 24 h at 4 °C. Then, the samples were washed with PBS and post-fixed in 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series and propylene oxide and embedded into Epon. For light microscopy, semithin sections (1 μm thick) were stained with toluidine blue. Thin sections of the cardia region were mounted on carbon-coated copper grids with Formwar film and stained with uranyl acetate and lead citrate and observed with 1200 JEOL electron microscope. Uninfected females, 10–12 days post blood meal, were processed in the same way and used as controls.

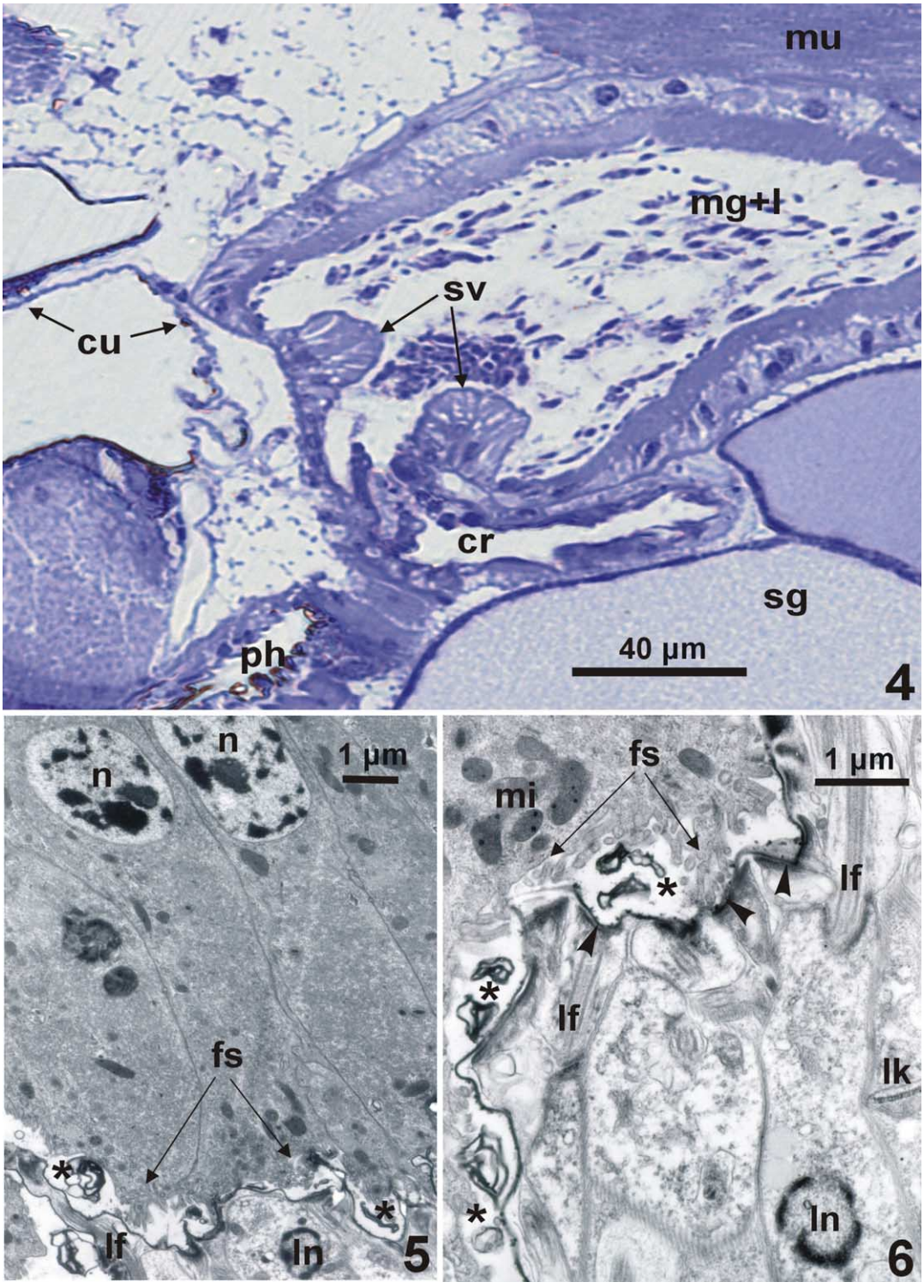
3. Results

In uninfected females, the stomodeal valve had a typical mushroom-like shape (Fig. 1). The apical ends of the cylindrical cells of the valve were rich in filamentous structures and covered by a thin electron-dense chitin layer (Figs. 2 and 3). Cells with these filamentous structures are present in the inner part of the valve close to the opening into stomodeum. Their position in the stomodeal valve (sv) is showed by arrows on Fig. 1.

Late stage infections of *Leishmania* in the sand flies *P. papatasi*, *P. duboscqi* and *L. longipalpis* resulted in high numbers of parasites which filled the whole gut lumen of



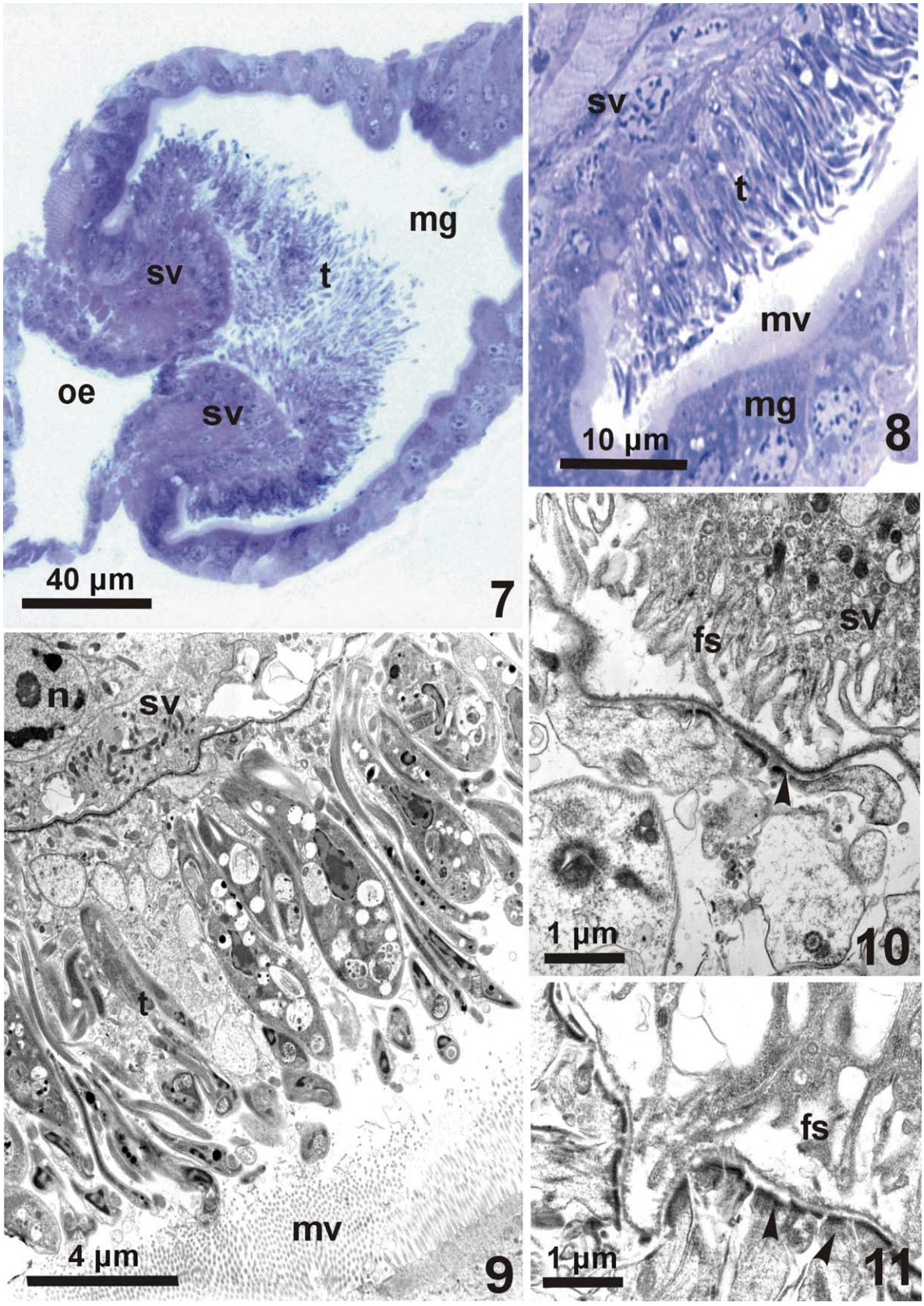
Figs. 1–3. Stomodeal valve of uninfected sand fly females. Semithin section of *Phlebotomus duboscqi* embedded in LR White resin and stained by toluidin blue. The position of filamentous structures in the stomodeal valve is showed by arrows (Fig. 1). Electron microscopy of the cylindrical cells in the inner part of the stomodeal valve of *P. duboscqi* (Fig. 2) and *Phlebotomus papatasi* (Fig. 3). Abbreviations: cr, crop; cu, cuticle; fs, filametous structures; mi, mitochondria; mg, midgut; mg + l, midgut containing Leishmania; mu, muscles; mv, microvilli of the thoracic midgut; n, nucleus of epithelial cells of the stomodeal valve; fl, Leishmania flagellum; lk, Leishmania kinetoplast; ln, Leishmania nucleus; oe, oesophagus; ph, pharynx; sg, salivary glands; sv, stomodeal valve; t, Trypanosoma; arrowheads, hemidesome-like plaques on parasite flagellum; *, degradation of the chitin layer.



Figs. 4–6. Stomodeal valve of sand fly females infected by *Leishmania* parasites. Semithin section of *Phlebotomus duboscqi* infected by *Leishmania major*. Sample is embedded in LR White resin and stained by toluidin blue. The position of degraded filamentous structures in the stomodeal valve is showed by arrows (Fig. 4). Electron microscopy of the cylindrical cells of the stomodeal valve in sand flies infected by *L. major* pathological changes of the filamentous structures and the chitin lining of *Phlebotomus papatasi* (Fig. 5) and *P. duboscqi* (Fig. 6). For abbreviations refer the caption of Figs. 1–3.

the cardia and anterior midgut. Some promastigotes were found in close contact with the midgut microvilli or with the cuticle lining of the valve, others were swimming freely in the midgut lumen. The stomodeal valve was permanently opened, its shape was changed and cylindrical cells appear to be reduced in size (Fig. 4).

Under the electron microscope, degenerative changes of the cylindrical cells were observed in all heavily infected



Figs. 7–11. *Culex pipiens quinquefasciatus* infected by *Trypanosoma* sp. Semithin sections of the stomodeal valve embedded in LR White resin and stained by toluidin blue (Figs. 7–8). Electron microscopy of the apical end of cylindrical cells and the chitin lining (Figs. 9–11). For abbreviations refer the caption of Figs. 1–3.

flies. The chitin layer was separated from the apical end of the cell and the filamentous structures were degraded. However, the nuclei and mitochondria of cylindrical cells appeared the same as in uninfected controls. The *Leishmania* promastigotes attached to the chitin layer of the valve by their flagella, the adhesions occurred by the formation of zonal hemidesmosome-like plaques. On some occasions, electron-dense debris of the chitin layer were observed between the apical end of the cells and the lining (Figs. 5 and 6).

Avian trypanosomes developing in *C. pipiens quinquefasciatus* caused the enlargement of the stomodeal valve of infected females; these changes were visible even during the dissections under the stereomicroscope. *Trypanosoma* epimastigotes were found attached to the stomodeal valve of *Culex* mosquitoes (Figs. 8 and 9) and, in contrast to the *Leishmania*–sand fly model, no free parasites were observed swimming in the lumen of mosquito cardia. Epimastigotes were orientated with their apical ends to the chitin lining of the valve and were attached by flagella forming hemidesmosome-like plaques. Destruction of the valve, the apical end of the cylindrical cells and their chitin lining appeared to be less pronounced than in *Leishmania* infections (Figs. 7–10).

4. Discussion

Our study showed that various trypanosomatid parasites colonizing the anterior part of the midgut of nematoceran Diptera used the same 'strategy', they block the cardia region and the stomodeal valve of the vector. Destruction of the valve or impairment of its function could facilitate the transmission of these parasites into their vertebrate hosts. In sand flies this is supposed to be a prevailing mechanism of *Leishmania* transmission (Schlein et al., 1992; Schlein, 1993). Data obtained in the *Culex–Trypanosoma* model showed that this specific feature of vector–pathogen interaction is similar to changes caused by late-stage infections of *Leishmania* in sand flies.

In sand flies, the damage occurring to the stomodeal valve during heavy *Leishmania* infections was first described by Schlein et al. (1992). They presented a picture drawn from sections and a scheme for the feeding mechanisms of *P. papatasi* showing the permanently opened valve and explaining the impaired ingestion of blood in females infected by *L. major*. They also reported various degrees of disruption of cylindrical cells of the valve, separation of individual cells from the tissue and a decrease in their volume or the loss of the cell membrane. In our study, some of these pathological changes were observed in semi-thin or ultra-thin sections. The valve of heavily infected females was opened and the cytoplasm of cylindrical cells was separated from the chitin layer. Similar detachment of the valve lining from the epithelial cells was also visible in electron micrographs presented by Walters et al. (1987, 1989b) and Walters (1993) in three *Lutzomyia* species, and we conclude that it is a general feature accompanying heavy *Leishmania* infections.

Filamentous structures at the apical end of cylindrical cells were observed in females of all models studied, including the *Culex* mosquitoes. They have not been described in previous ultrastructural studies; however, similar structures appear to be present in the micrographs of Walters (1993). The filaments connect the cell membrane to the inner layer of the chitin lining of the valve. We suggest that these structures are contractile and participate in the function of the stomodeal valve as a pump and/or valve. In the micrographs presented by Schlein et al. (1992) the filaments are not seen because of the orientation of the ultra-thin sections. They are present only in cells at the apical end of the valve where the valve closes the entrance into the midgut and where the cylindrical cells are needed to control the valve entrance. In infected sand fly females these filaments were separated from the chitin layer of the valve and were degraded.

Schlein et al. (1991, 1992) presumed that the chitinolytic enzymes of the parasite degraded the inner softer layer of the chitin of the stomodeal valve. Recent data obtained using *Leishmania* mutants differing in their production of chitinase (Rogers et al., unpublished) suggest that this enzyme really has an important role in the damage of the stomodeal valve. Such degradation of the chitin lining may cause its detachment from the cylindrical cells and expose the underlying tissues to the harmful effects of other parasite-secreted substances. Pathological changes observed in our experiments on the apical end of cylindrical cells, mainly the destruction of filamentous structures, suggest that *Leishmania* proteases such as leishmanolysin gp63 could be involved in this process.

In conclusion, the mechanical block of the stomodeal valve by the parasites embedded in the gel-like matrix resulted in partial dysfunction of the stomodeal valve and facilitates the transmission of the *Leishmania* and *Trypanosoma* parasites into the vertebrate host. In *Leishmania* infection this event is also facilitated by destruction of the chitin lining of the valve and by the degradation of the cylindrical cells, especially their apical ends with unique filamentous structures. We propose that the phenomenon of the 'blocked valve' may occur more generally in trypanosomatids transmitted by the bite of nematoceran Diptera.

Acknowledgements

We thank Dr P.A. Bates and Prof. R.D. Ward for valuable comments on the manuscript. The study was supported by the Grant Agency of Charles University (project 147/2002/B-Bio) and by the Ministry of Education of the Czech Republic (project 113100004 and FRVS 2356/2002).

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