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Ultrastructure of the salivary glands of non-infected and infected glands in *Glossina pallidipes* by the salivary glands hypertrophy virus

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ABSTRACT

Light, scanning electron, and transmission electron microscopy analyses were conducted to examine the morphology and ultrastructure of the salivary glands of *Glossina pallidipes*. Three distinct regions, each with a characteristic composition and organization of tissues and cells, were identified: secretory, reabsorptive and proximal. When infected with the salivary gland hypertrophy (SGH) virus, glands showed a severe hypertrophy, accompanied by profound changes in their morphology and ultrastructure. In addition, the muscular fibers surrounding the secretory region of the glands were disrupted. The morphological alterations in the muscular tissue, caused by viral infection, could be an important aspect of the pathology and may shed light on the mode of action of the SGH virus. Results were discussed with regard to the potential effect of viral infection on normal salivation and on the ability of infected tsetse flies to transmit a trypanosome parasite.

1. Introduction

Tsetse flies (Diptera: Glossinidae) are obligate blood feeding insects and important disease vectors transmitting different pathogenic trypanosome species that cause human sleeping sickness and livestock trypanosomiasis in Africa. Trypanosomes of the *Trypanosoma brucei* group, including the two human-pathogenic subspecies *T. b. gambiense* and *T. b. rhodesiense*, have to go through a complex developmental cycle in the alimentary tract and the salivary glands of the tsetse fly (Van Den Abbeele et al., 1999). Thus, the salivary glands play an important role in the transmission of the parasite.

Many species of tsetse flies are infected with a virus that causes salivary gland hypertrophy (SGH). Salivary Gland Hypertrophy Viruses (SGHVs) have been found and studied not only in tsetse flies but also in house flies, *Musca domestica* (Lietze et al., 2011). Recently, the genomic organization of SGHVs was determined and, on the basis of the available morphological, (patho)biological, genomic, and phylogenetic data, it was proposed that these viruses are members of a new virus family, named Hytrosaviridae (Abd-Alla et al., 2009). These hytrosaviruses are rod-shaped, contain large circular double-stranded DNA genomes, replicate in the nuclei of salivary gland cells in adult flies causing distinct tissue hypertrophy, and reduce fertility of their hosts (Lietze et al., 2011).

Our study focused on *Glossina pallidipes*, a species in which the first identification of virus particles, associated with SGH symptoms, dates to the 1970s (Jaenson, 1978). The *G. pallidipes* SGHV (GpSGHV), in addition to infecting the salivary glands, has been reported to replicate in the female milk gland as well as in gonadal tissues, resulting in testicular degeneration and ovarian abnormalities (Sang et al., 1999, 1998). Electron microscopic observations of virus particles, either in thin sections of hypertrophied salivary glands or from sucrose density gradient-purified, negatively stained preparations, showed enveloped bacilliform virions. The pathology of infected cells and effects on various organelles in the salivary glands have been documented (Jura et al., 1989; Kokwaro et al., 1991, 1990; Otieno et al., 1980). However, certain aspects of the interaction between the virus and host cells need further study, such as the cytopathology induced in the muscle cell layer or sheath covering the salivary glands.

Therefore, the purpose of this research was to describe the overall structure of both non-infected and infected salivary glands and their morphological and ultrastructural differences, to highlight potential viral effects on the glands' physiology, and to better understand the mode of action of SGHV in tsetse flies. Furthermore, this study may shed light on the possible relationship between the observed structural and assumed functional changes of the salivary glands and how viral infection may affect the role of tsetse flies as vectors of trypanosomes.

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2. Materials and methods

2.1. Animals

Pupae of *G. pallidipes* were received from a laboratory colony maintained in the Insect Pest Control Laboratory of the International Atomic Energy Agency (IAEA) in Vienna, Austria. These pupae were maintained at 24 °C, 70% relative humidity and a photoperiod of 12 h as described in previous studies (Abd-Alla et al., 2007; Feldmann, 1994; Gooding et al., 1997). Adults were fed with sugar solution and their average survival was 10 days. Specimens of both sexes of the adult insects were dissected to obtain samples of healthy salivary glands and virus-infected hypertrophied glands.

2.2. Light microscopy

Samples of salivary glands, infected and non-infected, were dissected in PBS and immediately observed using a computerized image analysis system, which included a Zeiss light microscope (Axiophot) equipped with a video color camera (Axio Cam MRC, Arese, Milano-Italy) and imaging software (KS 300 and AxioVision).

2.3. Scanning electron microscopy (SEM)

For the SEM investigation, infected and non-infected salivary gland samples were fixed for one night in 2.5% glutaraldehyde in 0.1 M cacodylate 3% sucrose buffer pH 7.2. Subsequently, the material was washed in 0.1 M cacodylate buffer overnight and then post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer. The specimens were dehydrated through a series of steps in acetone at progressively increasing concentrations (from 50% to 100%). The dehydrated material was dried with liquid carbon dioxide using a critical point dryer (Balzer Union CPD 020), mounted on a special sample holder, and coated using a gold evaporator (Balzer Union MD 010). So treated, the samples were observed under a Jeol JSM 5200 microscope.

2.4. Transmission electron microscopy (TEM)

For the TEM survey, samples of infected and non-infected salivary glands were fixed and dehydrated as described for the SEM analysis and then infiltrated in Epon resin (TAAB, England). Thin sections were cut with Reichert Ultracut and LKB Nova ultramicrotomes. Semithin sections (1 μm thick) collected on slides were stained with toluidine blue and then observed with Zeiss Axiophot light microscopy. Ultra-thin (thickness of 60–80 nm) sections were collected on copper grids, stained with uranyl acetate and lead citrate and observed at 120 kV using a Jeol JEM EX II transmission electron microscope, equipped by a Veleta CCD camera (Olympus) and the pictures where processed by iTEM software.

3. Results

The salivary glands of *G. pallidipes* consisted of two tubular, thin and transparent structures that extended into the abdomen on either side of the gut. In the normal and healthy salivary glands, each paired gland showed three distinct regions: secretory, reabsorptive, and proximal. This last portion continued in a duct joining a common salivary duct (Fig. 1A). The external morphology of healthy salivary glands was investigated by light microscopy and SEM. The distal, secretory region extended from the abdomen into the thorax and was surrounded by a thick muscle tissue with evident longitudinal muscle fibers (Fig. 1D and E), whereas the outer surfaces of the reabsorptive and proximal regions were devoid of a muscular coat (Figs. 1A–C).

The infection with SGH virus greatly enlarged the salivary glands, and this hypertrophy was uniform over the entire length of the gland. In fact, each individual salivary gland was affected equally, increased in diameter, and appeared whitish and pale, thus being easily distinguishable from non-infected and transparent glands (Fig. 2A). However, hypertrophy primarily affected the secretory region of the glands, which filled most of the abdominal cavity and became entangled with fat body and tracheae (Fig. 2A). Scanning electron micrographs revealed an evident alteration of the muscular tissue in the hypertrophied salivary glands; the longitudinal muscle fibers lost their organization and arrangement and, in several places, were disrupted (Fig. 2D and E).

Figs. 3 and 4 show and compare transmission electron micrographs obtained from the secretory regions of normal and hypertrophied salivary glands, respectively. Both light microscopy and TEM examination revealed that the secretory region of the normal gland was characterized by a muscular coat surrounding the gland, an epithelium consisting of a single layer of cells, and a central lumen (Fig. 3A and B). TEM observations of cross sections of this region showed that the external muscle tissue was composed of large muscle cells, each showing numerous myofibrils (Fig. 3C). The secretory cells were separated from the muscle by a basement membrane and contained an extensive rough endoplasmic reticulum, many Golgi complexes, numerous mitochondria, some of which had electron-dense granules, and a large number of secretory granules (Fig. 3D, F and G). Especially in the apical area of the epithelium, adjacent cells were in contact along their lateral plasma membranes by septate desmosomes (Fig. 3E). In addition, the secretory cells showed microvilli extending into the gland lumen and involved in the release of secretion (Figs. 3E and H). The lumen contained an electron-dense matrix with numerous electron-opaque filaments (Fig. 3E).

In the secretory region of the hypertrophied glands, infected by SGH virus, the muscular coat surrounding the gland underwent profound ultrastructural changes (Fig. 4A–C). Transmission electron micrographs showed the presence of vacuoles in the muscle cells, even in the region contacting the underlying basement membrane (Fig. 4C). Owing to the remarkable degree of hypertrophy, this basement membrane had an irregular and not well defined structure, appeared stratified and thicker than the same region of the healthy salivary glands, and showed evident changes in the relationship with the muscle cells (Fig. 4C). Moreover, cells of the glandular epithelium were highly vacuolated (Fig. 4A and B), cell junctions were no longer visible, and the gland enlargement was caused by cellular proliferation of the secretory cells (Fig. 4D), resulting in an abnormal multilayered epithelium and a reduced gland lumen (Fig. 4A). In certain areas, the proliferating cells lost contact with the degenerated basement membrane because of the vacuolization of the cytoplasm (Fig. 4C).

Numerous virus particles were scattered in the nuclei and in the cytoplasm of secretory cells (Fig. 4E and F). Fig. 4E depicts the morphological differences of the virus in the nucleus and in the cytoplasm; in the nucleus, the virus replicated and assembled its nucleocapsid, and with the transition into the cytoplasm, the viral particles further developed. The high-magnification micrograph in Fig. 4G shows longitudinal and cross sections of the SGH virus particles in the cytoplasm of the secretory cells.

In the normal salivary glands, with the transition from the secretory to the reabsorptive and then to the proximal region, the diameter of the gland decreased (Fig. 2A). The reabsorptive region was localized in the thorax of the insect, and at the electron microscope level this region was characterized by an external basal lamina with a smooth outside surface and underlying epithelial