Comparative ultrastructural characterization of African horse sickness virus-infected mammalian and insect cells reveals a novel potential virus release mechanism from insect cells

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African horse sickness virus (AHSV) is an arbovirus capable of successfully replicating in both its mammalian host and insect vector. Where mammalian cells show a severe cytopathic effect (CPE) following AHSV infection, insect cells display no CPE. These differences in cell death could be linked to the method of viral release, i.e. lytic or non-lytic, that predominates in a specific cell type. Active release of AHSV, or any related orbivirus, has, however, not yet been documented from insect cells. We applied an integrated microscopy approach to compare the nanomechanical and morphological response of mammalian and insect cells to AHSV infection. Atomic force microscopy revealed plasma membrane destabilization, integrity loss and structural deformation of the entire surface of infected mammalian cells. Infected insect cells, in contrast, showed no morphological differences from mock-infected cells other than an increased incidence of circular cavities present on the cell surface. Transmission electron microscopy imaging identified a novel large vesicle-like compartment within infected insect cells, not present in mammalian cells, containing viral proteins and virus particles. Extracellular clusters of aggregated virus particles were visualized adjacent to infected insect cells with intact plasma membranes. We propose that foreign material is accumulated within these vesicles and that their subsequent fusion with the cell membrane releases entrapped viruses, thereby facilitating a non-lytic virus release mechanism different from the budding previously observed in mammalian cells. This insect cell-specific defence mechanism contributes to the lack of cell damage observed in AHSV-infected insect cells.

INTRODUCTION

A common feature of arthropod-borne viruses (arboviruses) is their ability to successfully infect different species, evident by the production of infectious progeny viruses from both mammalian hosts and insect vectors. Distinct differences are, however, observed in the effect of the viral infection on these species, with hosts exhibiting a range of clinical signs, whereas vectors remain unaffected (Mellor et al., 2000, 2009). African horse sickness virus (AHSV) is an arbovirus and member of the family Reoviridae and genus Orbivirus, of which the closely related bluetongue virus (BTV) is the orbivirus prototype. AHSV is the aetiological agent of African horse sickness (AHS), a World Organization for Animal Health (OIE) listed notifiable disease of equids, with infection resulting in an equine mortality rate of up to 95% (Coetzer & Guthrie, 2004). Both AHSV and BTV are transmitted by Culicoides midge species. The occurrence of AHS and bluetongue is dependent on the distribution of the insect vectors, and recently changes in climatological factors such as global warming have resulted in the spread of these diseases to previously non-endemic areas (Gould & Higgs, 2009).

Orbiviruses are non-enveloped icosahedral viruses composed of a segmented dsRNA genome enclosed by a dual-layered protein capsid (Mellor & Hamblin, 2004; Mertens & Diprose, 2004). The ten dsRNA genome segments encode seven structural (VP1–VP7) and four non-structural (NS1–NS4) proteins (Ratinier et al., 2011; Roy et al., 1994). The ~70 nm spherical virion consists of VP2 and VP5 forming the outer layer, and VP3 and VP7 that form the ~50 nm core particle together with minor core proteins VP1, VP4, VP6 and ten dsRNA genome segments. Following virus adsorption and endocytosis, the outer coat proteins are removed and the core particle released into the cytoplasm. Transcription, translation and progeny virus assembly occur within the cytoplasm of infected cells. The most prominent manifestations of AHSV infection are the formation of large granular viral inclusion bodies (VIBs),
and the assembly of thin tubular structures in the cytoplasm (Huismans & Els, 1979; Uitenweerde et al., 1995). The matrix of the VIBs is composed of non-structural protein NS2, and these viral factories function as a scaffold for viral replication and assembly. The function of the tubules remains largely unknown, but they are comprised exclusively of multimers of the non-structural protein NS1. Following assembly at the periphery of the VIB, mature progeny viruses are transported to the plasma membrane for release by either a lytic or a budding mechanism. The two progeny viruses are transported to the plasma membrane for release by either a lytic or a budding mechanism. The two non-structural proteins NS3 and NS3A, encoded from in-frame overlapping reading frames of genome segment 10, are involved in virus release (Stoltz et al., 1996).

In AHSV-infected cell culture, mammalian cells exhibit varying types of morphological changes culminating in death (Meiring et al., 2009), whereas insect cells do not show any observable cytopathic effect (CPE) (Stassen et al., 2012). It is postulated that lytic release occurs in infected mammalian cells, whereas only a non-lytic form of release such as budding takes place in insect cells. Lytic release is characterized by cell-membrane damage and disruption in the area of virus release, while budding of these non-enveloped viruses represents the process whereby a virus protrudes from the cell membrane and temporarily acquires a membrane envelope while leaving the plasma membrane intact (Beaton et al., 2002; Han & Harty, 2004; Owens et al., 2004).

One strategy of investigating this hypothesis is by means of ultrastructural characterization of infected cells. To date, electron microscopy of AHSV has been focused on descriptions of life-cycle events in mammalian cells and tissues (Breese et al., 1969; Carrasco et al., 1999; Gómez-Villamandos et al., 1999), with no characterization of AHSV-infected insect cells. Thus, it remains unclear whether the type of release mechanisms employed, other aspects of the AHSV life-cycle, or the host cell response, could contribute to the absence of CPE and cell death in insect cells. This study represents the first ultrastructural comparison of AHSV morphogenesis in mammalian and insect cells. We applied an integrated microscopy approach to study both these cell types. Based on our results, we propose an insect cell-specific defence mechanism that facilitates non-lytic virus release. The release mechanism is different from the budding previously observed in mammalian cells and contributes to the lack of cell damage in AHSV-infected insect cells.

**RESULTS**

**Cell morphology and mechanical properties of mock-infected and AHSV-infected Vero and KC cells**

Upon AHSV infection, mammalian cells usually show severe CPE within the first 24–48 h, whereas Culicoides-derived KC cells remain viable for more than 2 weeks. Atomic force microscopy (AFM) was used to investigate cell-surface morphology and to quantify changes in nanomechanical properties of the plasma membranes of infected cells. Cells were fixed on coverslips in an attempt to immobilize any rare virus-induced events at the cell membrane and to prevent dislodging of non-fixed cells by the AFM tip. It has previously been established that the fixation conditions employed here would not significantly impact on the structure or dimensions of the cells (Butt et al., 1990).

AFM imaging was done on 50 mock-infected control cells and 50 AHSV-infected mammalian Vero cells (Fig. 1a). Gross cell morphology was imaged from the peak force error channel (left panel) and three-dimensional (3D) height sensor measurements (middle panel), which were obtained by scanning an oscillating probe tip over the sample surface to record force-distance curves and sample height variations, respectively. Mock-infected Vero cells (Fig. 1a, top panels) were elongated, 40–65 μm in size and displayed a smooth membrane topography. Infected cells (Fig. 1a, bottom panels) were rounder, and exhibited an irregular topography with multiple protrusions on the cell surface. These protrusions ranged from 300 nm to 5 μm in diameter. The degree of membrane destabilization, and area exhibiting loss of integrity, were assayed using the deformation channel image (Fig. 1a, right panel). The deformation values were determined by exertion of a small but constant force on the cell surface by the microscope probe tip, and recording of the distance that the tip could deform the membrane at each point. Mock-infected cells displayed an average of 27.4 ± 15.6 nm of deformation, with an approximately equal magnitude at any point of the cell surface. However, in AHSV-infected cells, the mean deformation increased by 50 % to 41.5 ± 23.5 nm. Areas of the surface that displayed the highest deformation values (Fig. 1a, yellow colouring) corresponded to the membrane protrusions visualized from the height sensor images. These membrane protrusions had deformation values approximately 2.5 times higher than other portions of the cell membranes, with values of up to 90 nm.

Cell morphology and membrane deformation changes can result from virus-induced modifications of the cellular cytoskeleton network (Cai et al., 2010). To investigate the effect of AHSV on the cytoskeleton, Vero cells were fixed, labelled with anti-tubulin markers and analysed by confocal laser-scanning microscopy (Fig. 1b). These images confirmed the elongated shape of mock-infected cells versus rounding of virus-infected cells described from the AFM profiles, and a slight redistribution of the microtubule network towards the cell periphery following AHSV infection; however, overall the cells exhibited similar tubulin fibre distribution patterns. There was no sign of total destruction of the normal microtubule network. Therefore, AHSV infection did not induce major alterations to the cytoskeleton, but impacted on membrane integrity and cellular morphological and mechanical properties by another mechanism.

To investigate the response of insect cells to AHSV infection, 50 mock-infected and 50 infected KC cells were analysed by AFM (Fig. 1c). Mock-infected cells (top panel)
had a diameter of 7 to 20 μm, were round to slightly elongated in shape with a smooth membrane topography lacking any surface protrusions, and displayed a mean deformation of 76.9 ± 21.1 nm. One to three circular indentations or cavities, ranging in diameter from 500 nm to 2.5 μm, were present on the cell surface of 20% of mock-infected cells. Virus-infected cells (Fig. 1c, bottom panel) were similar to the control cells both in terms of morphology and with respect to their membrane deformation values of 80.2 ± 18.9 nm. The only difference was that, in the infected sample, there was a doubling of the number of cells that contained the cavities in their plasma membranes, with 42% of the cell population displaying cavities. The circular cavities in mock-infected and infected cells were similar in size. Scanning electron microscopy (SEM) images of KC cells (Fig. 1d) confirmed the presence and size of these cavities identified by AFM.

Comparative ultrastructural characterization of the virus life cycle in AHSV-infected mammalian and insect cells

To examine the ultrastructural pathology of AHSV infection, two insect cell lines (KC and mosquito-derived C6/36 cells) and mammalian Vero cells were infected with AHSV, cryofixed and visualized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The TEM images showed the formation of viral capsids and the budding of virus particles at the cell surface. SEM images revealed the formation of circular indentations on the cell membrane, which were attributed to the viral infection process. The comparative ultrastructural analysis provided insights into the virus life cycle and the mechanism of membrane deformation induced by AHSV infection.
microscopy (TEM). Infected insect cells (Fig. 2) were similar in cell size, shape, cytoplasmic density and organelle distribution to mock-infected cells, but in addition displayed all the hallmarks of AHSV infection described previously in mammalian cells. In a single section plane, up to three amorphous VIBs of varying sizes could be detected in the cytoplasm of the majority of infected cells (Fig. 2a). Immunogold labelling confirmed that they were composed of non-structural protein NS2 (Fig. 2a, insert). Within the VIB protein matrix, dense circular virus core particles of ~50 nm were seen. Mature ~70 nm spherical virions, which result from the assembly of the two outer capsid proteins around the core, were usually present at the VIB periphery. Multiple virus particles were often arranged in a precise manner into a paracrystalline array (PCA) (Fig. 2b). Even though the PCAs could be found directly adjacent or in close proximity to VIBs, immunogold labelling indicated that they contained no NS2 protein (results not shown). Virus-specific tubules (Fig. 2c) were abundant within the cytoplasm as well as in the nuclei. These ~23 nm wide, hollow tubules reached a length of up to 3 μm and were composed of non-structural protein NS1 (Fig. 2c, insert). Rectangular crystalline structures of varying size were observed within the cytoplasm (Fig. 2d), or rarely within nuclei. These structures were similar to the ‘cytoplasmic inclusions’ or ‘lamellar structures’ first described by Breese in 1969 (Breese & Ozawa, 1969; Breese et al., 1969). Up to three of these crystalline particles, ranging from 75 to 650 nm in width and 800 nm to 5 μm in length, with no specific width-to-length ratio, were found in the majority of cells. Strong and specific labelling with an anti-VP7 antibody (Fig. 2d, insert) confirmed that these structures represent the hexagonal VP7 crystals that can be purified from AHSV-infected cells (Burroughs et al., 1994). Upon higher magnification, it was observed that these particles had a sheet-like appearance (Fig. 2e), corresponding to the highly ordered two-dimensional crystalline lattice structure described for the purified VP7 crystals (Burroughs et al., 1994). Occasionally, mature virions or NS1 tubules were observed in close association with the VP7 crystals, but this was not deemed to be of functional significance.

A novel structure was observed in insect cells that has not been identified previously in AHSV-infected mammalian cells. In both KC and C6/36 cells, a number of large, circular, membrane-bound vesicle-like structures ranging in size from 500 nm to 5 μm were present. In mock-infected control cells, these vesicles were either empty (Fig. 3a) or contained some cellular debris (Fig. 3b). However in infected cells, these structures usually contained viral material (Fig. 3c, d). We observed multiple mature virus particles and immature cores within these vesicles (Fig. 3c, einsert) as well as VP7 crystalline structures and NS1 tubules (Fig. 3e, f). The virus-specific nature of all of these

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**Fig. 2.** Ultrastructure of AHSV-infected KC cells illustrating structures associated with viral replication. (a–e) KC cells were infected with AHSV at an m.o.i. of 0.5, processed at 7 days p.i. and fixed by high-pressure freezing and freeze-substitution. A VIB, paracrystalline array (PCA), tubules (Tub) and crystalline structures (Cr) are indicated. (Inserts) Immunogold labelling with anti-NS2 (a), anti-NS1 (c) and anti-VP7 (d) antibodies illustrates the protein component of the VIBs, tubules and crystalline structures. Bars, 2 μm (a); 500 nm (b–e and inserts).
structures was confirmed by immunogold labelling (not shown). Interestingly, labelling with anti-NS1 or anti-NS2 antibodies gave significantly stronger positive signals within the vesicles than in the cytoplasm, even when no tubules or VIBs could be discerned. This could indicate some preferential inclusion of cytoplasmic material containing foreign proteins into the vesicles. These vesicles were sometimes observed in very close proximity to the plasma membrane (Fig. 3g). Similar vesicle-like structures were documented in BTV-infected Culicoides cells (Celma & Roy, 2011); however, the authors only observed multiple fully packaged viruses, but no other viral material, inside the vesicles.

Orbivirus release from insect cells has long been postulated to be primarily non-lytic, as virus infection results in no observable CPE. In an attempt to observe this release, we meticulously screened more than 300 cells but were never able to capture any budding-type event similar to that observed in mammalian cells (see below). We even cultured and processed the cells directly in 200 μm diameter cellulose microcapillary tubes, as we recently reported that this reduces mechanical disruption and increases the chance of observing virus release (Venter et al., 2012), but with no success. However, we visualized extracellular viruses, present either as individual particles or as clusters of aggregated viruses, between neighbouring

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**Fig. 3.** Ultrastructure of C6/36 and KC cells illustrating intracellular vesicles and virus release. (a, b) Mock-infected C6/36 cells harbouring empty vesicles (arrows) or occasional vesicles containing some cellular material (arrowheads). (c–h) Cells were infected with AHHSV at an m.o.i. of 0.5, processed at 7 days post-infection and fixed by high-pressure freezing and freeze-substitution. (c) Large vesicle-like structures in C6/36 cells contained core and virus particles. Insert: core particles (C) and virions (V) could be distinguished based on size and presence of the outer capsid. (d) Large vesicle-like structure containing virus material. (e, f) Enlargement of the demarcated areas from (d), indicating crystalline structures (Cr) and tubules (Tub). (g) Vesicle adjacent to a plasma membrane. (h) Extracellular clusters of aggregated viruses (asterisks), and individual particles (V) between intact plasma membranes of infected KC cells. Bars, 2 μm (a–c, e); 200 nm (insert); 500 nm (d, f–h).
infected cells with intact plasma membranes (Fig. 3h). These virus particles were not remnants of the viral inoculum, as samples processed 1 h post-infection (p.i.) did not exhibit any extracellular viruses (results not shown). From these data, we propose that AHSV is rarely or never released directly from the insect cell cytoplasm by budding, but rather by fusion of the large virus-containing vesicles with the plasma membrane.

For comparison with the insect cells, we also viewed mammalian cells by TEM (Fig. 4). Previous reports on the ultrastructure of AHSV-infected mammalian tissue (Breese et al., 1969; Carrasco et al., 1999; Gómez-Villamandos et al., 1999; Lecatsas & Erasmus, 1967) used conventional chemical fixation. However, sample preparation by cryofixation through high-pressure freezing and freeze-substitution, as used here, yields increased sample quality and membrane preservation (Studer et al., 2001), which can be crucial for TEM visualization of plasma-membrane virus release events (Hawes et al., 2007). From the micrographs, the gross morphological structure of infected Vero cells correlated with that observed by AFM. The cells did not retain their elongated shape but became rounder and exhibited multiple membrane protrusions (Fig. 4a). The other typical virus-induced structures were also present, e.g. VIBs, PCAs, VP7 crystals and NS1 tubules (not shown). Based on our description of the vesicle-like structures containing virus material in insect cells, we carefully examined mammalian cells for the presence of similar components. In about 5% of cells, there were small vesicles, ranging in size from 100 to 500 nm that contained one to five virus particles (Fig. 4b, c). Occasionally, merging of such a vesicle with the membrane was observed (Fig. 4d). We postulated that, at this late stage of the infection cycle, i.e. 48 h p.i., it represented the release of a mature virion, rather than an entry event. These vesicles did not contain any other cytoplasmic or viral material. However, the bulk of virus release appeared to be through a lytic process, where extensive membrane damage occurred in close proximity to extracellular virus particles (Fig. 4e). In less than 2% of cells, we recorded a non-lytic budding-type release, where an individual virus particle protruded through the membrane without any visible membrane damage (Fig. 4f).

DISCUSSION

The exact nature of the cellular response to AHSV infection, the differences in induction of a cell death response in different cell types and the contribution of AHSV morphogenesis to these is not fully understood. This study represents an ultrastructural comparison of AHSV-infected mammalian and insect cells using AFM, SEM, TEM and confocal fluorescence microscopy.

Fig. 4. Ultrastructure of AHSV-infected Vero cells. Vero cells were infected with AHSV at an m.o.i. of 5, processed at 48 h p.i. and fixed by high-pressure freezing and freeze-substitution. (a) Cells had irregular plasma membranes containing multiple membranous protrusions (arrowheads). (b, c) Small intracellular vesicles containing up to five virus particles (arrows). (d) A single virus-containing vesicle merging with the plasma membrane. (e) Virus particles (arrows) in close proximity to a damaged membrane (asterisks). (f) Virus particles budding from the cell surface. Bars, 4 μm (a); 500 nm (b–f).
Initially, AFM was used to examine cell morphology in response to viral infection. This is the first study using AFM for orbiviruses. The AFM profiles of AHSV-infected Vero cells revealed a highly uneven plasma membrane topology and an increased deformability in comparison with mock-infected controls. The change in membrane integrity was not uniform across the cell surface. The areas where the largest cell-surface protrusions or membrane blebs were identified from the 3D scans also showed the biggest deformation ability when probed by the microscope tip. This indicated that the membrane changes were not a generalized response to the release of chemical inflammatory mediators, for example (Carrasco et al., 1999). We also ruled out the possibility that the surface disruption was the result of underlying modifications to the cytoskeletal structure upon virus infection, such as that induced by rotavirus replication in cell culture (Yang & McCrae, 2012), because the microtubule network organization in AHSV-infected cells showed only minor redistribution.

These surface protrusions could be membrane blebs formed as a result of the infected cells undergoing apoptosis (Stassen et al., 2012), similar to what was observed by SEM for BTV (Nagaleekar et al., 2007). Alternatively, these bulges could be areas where localized membrane destabilization has occurred prior to virus egress by lytic release. Immunofluorescence microscopy showed that, in BTV-infected cells, the viral outer capsid protein VP5, together with non-structural protein NS3, co-localize in small patches at the plasma membrane (Bhattacharya & Roy, 2008). NS3 is capable of binding to both cellular membrane trafficking proteins and to the viral capsid to mediate virus release (Beaton et al., 2002; Wirblach et al., 2006), and AHSV NS3 is present in membrane fragments close to areas of lytic virus release (Stoltz et al., 1996). What remains unclear in AHSV-infected cells is the link between the virus-induced membrane permeability changes and the membrane deformation observed here. We showed previously that NS3 can act as a viroporin in the AHSV life cycle by altering membrane permeability and facilitating virus release; however, the degree of membrane permeabilization induced by different AHSV strains did not correspond to the level of CPE observed (Meiring et al., 2009). The localization of AHSV NS3 and/or capsid proteins to the regions of membrane protrusion could be investigated by modifying the AFM tip with addition of virus-specific antibodies.

TEM of Vero cells confirmed the major disruption of cell morphology and plasma membrane integrity following AHSV infection. Cells exhibited characteristics of both apoptosis, such as chromatin condensation, membrane blebbing and apoptotic bodies, and of necrosis, such as cell rounding and swelling, and loss of membrane integrity (Galluzzi et al., 2012). We verified that virus release from a mammalian cell line is mostly via a lytic process, and also occasionally from a budding-type event. We also for the first time described the presence of small cytoplasmic vesicles containing one or a few virions. Similar structures have been observed in BTV-infected cells, and were proposed to be either endosomal entry events at early times post-infection (Eaton et al., 1990) or budding of virions into cytoplasmic vesicles that occurred late in the course of infection (Brewer & MacLachlan, 1994). Both BTV and AHSV NS3 can interact with Tsg101 (Wirblach et al., 2006), a member of the ESCRT protein complex involved in governing the biogenesis/degradation of multivesicular bodies (MVBs) (Babst, 2005). MVBs can traffic either to lysosomes where they are subjected to proteosomal degradation (termed ‘degradative MVBs’) or to the plasma membrane where they fuse with the membrane and release their contents into the extracellular space (termed ‘exocytic MVBs’) (Mathivanan et al., 2010). Based on the late times after infection that they were present, and the presence of multiple viruses in these vesicles, we postulate that the observed vesicles function as exocytic MVBs that allow temporary storage for completely packaged viruses and serve as an additional mechanism to facilitate virus release.

The AFM and SEM profiles of the AHSV-infected KC cells showed unchanged morphology and mechanical properties when compared with uninfected cells. This correlated with the previously documented lack of insect cell damage following BTV and AHSV infection (Stassen et al., 2012; Wechsler & McHolland, 1988). However, we observed a 100 % increase in the prevalence of large circular cavities present on the cell surface following virus infection. TEM imaging of cell sections identified membrane-bound vesicular compartments in the cytoplasm of both uninfected and AHSV-infected insect cells, corresponding in size to the cavities. We therefore propose that these cavities imaged by AFM represent the merging of these cytoplasmic vesicles with the plasma membrane, thereby enabling the release of their content, and that AHSV infection increases either the number of these vesicles or the rate at which they fuse with cell membrane.

In contrast to the normally empty status of the vesicles in mock-infected cells, those in AHSV-infected cells usually contained viral proteins or virus particles. This might thus represent a cellular defence mechanism, activated by viral infection, whereby any foreign material could be stored within these vesicle-like structures for subsequent expulsion. It is unlikely that AHSV replication occurred within the vesicles, as VIBs were never observed within them. It is not known whether these vesicles are autophagosomes. Autophagy is a cellular process that targets cytoplasmic components for autodigestion but is also important in innate and adaptive immunity and in controlling intracellular microbes (Deretic & Levine, 2009). A number of viruses have been shown to subvert the autophagy response by either promoting virus replication or by enabling non-lytic viral release (Lin et al., 2010). Autophagy was also proven to play a direct antiviral role against the mammalian pathogen vesicular stomatitis virus in the insect Drosophila (Shelly et al., 2009). Autophagosomes are normally distinguished by the presence of a double-layered membrane thought to be primarily derived from
the endoplasmic reticulum (Juhasz & Neufeld, 2006). Although some of the vesicles we observed contained a double membrane (results not shown), the majority of these structures were enclosed by a single-layered membrane. Due to the fast turnover of the fusion of autophagosomes with lysosomes, which results in inner-membrane loss, and the prolonged infection times utilized here, these single-membrane structures could well represent autolysosomes (Deretic & Levine, 2009).

The difficulty in visualizing active virus release events from infected insect cells confirmed our lack of understanding of virus transport in and release from these cells. The sequestration of a large fraction of the infectious AHSV particles into a vesicular compartment in insect vector cells could also account for the absence of CPE in cell culture and the lack of any deleterious effect of viral infection on the Culicoides vector. Our observation of aggregated virus particles outside infected insect cells that showed no membrane damage supported the view that viruses were being released en masse via a non-lytic release mechanism. Similar virus-containing vesicles were described in BTV-infected insect cells; however, there the simultaneous expression of both NS3 and NS3A was required for vesicle formation (Celma & Roy, 2011). Mutant BTV viruses lacking either NS3 or NS3A showed attenuation of viral replication, absence of vesicles, virus particles scattered throughout the cytosol and reduction of virus release. As no budding from the plasma membrane was ever observed from BTV-infected KC cells (Celma & Roy, 2011), the mechanism of release of these mutant viruses from insect cells remains unclear. Peruvian horse sickness virus, another member of the genus Orbivirus, was associated with vesicles in C6/36 cells (Attoui et al., 2009), but these were small vesicles containing only one or two virus particles. Rice dwarf virus (RDV), also a member of the genus Reoviridae that is able to replicate in plants and insects, utilizes vesicles in insect vector cells morphologically very similar to those we have described here (Wei et al., 2008). It was proposed that RDV particles are assembled at the periphery of the viroplasm (the equivalent of the AHSV VIBs), engulfed by the vesicular compartments and subsequently released upon fusion of these compartments with the cell membranes.

The outcome of this study is the first ultrastructural comparison between AHSV-infected insect and mammalian cells. We hypothesize that the lack of cell death observed in infected insect cells is at least partly due to a distinct cellular response resulting in the accumulation of viral material into vesicle-like structures. These vesicles then merge with the plasma membrane to release all of the contents, including virus particles, thereby facilitating a non-lytic form of release. This insight into the insect cell response to AHSV infection should be expanded by analyses focusing on AHSV NS3 distribution and function, vesicular trafficking and host autophagy response pathways.

**METHODS**

**Cells and viruses.** Vero cells (ATCC CCL-81) were cultured in the presence of Eagle’s minimal essential medium (MEM) supplemented with 5% FCS, antibiotics and antifungals (penicillin, streptomycin and fungizone) at 37 °C in 5% CO2. KC cells (derived from Culicoides variipennis) obtained from the Onderstepoort Veterinary Institute (OVI, South Africa), were cultured at 28 °C in modified Schneider’s Drosophila medium supplemented with 10% FCS, antibiotics and antifungals. C6/36 cells (Aedes albopictus; ATCC CRL-1660) were cultured at 28 °C in MEM supplemented with 10% FCS, 1% pyruvate, antibiotics and antifungals.

AHSV serotype 3 (AHSV-3) was obtained from the OIE Reference Laboratory (OVI, South Africa). AHSV-3 stocks were generated by infecting Vero cells at a low m.o.i., harvesting the cells when sufficient CPE was exhibited and storing at 4 °C. Virus titres were determined by plaque assay as described previously (Meiring et al., 2009).

Cells were cultured either on glass coverslips until ~75% confluent for AFM, SEM and confocal imaging or as a monolayer in culture flasks for TEM visualizations as described previously (Venter et al., 2012). KC and C6/36 cells were infected with AHSV-3 at an m.o.i. of 0.5 p.f.u. per cell and Vero cells at an m.o.i. of 5. The virus inoculum was removed after 1 h of adsorption and replaced with fresh medium. Vero cells were processed at 24–48 h p.i., and KC and C6/36 cells at 7 days p.i.

**AFM analysis.** Coverslips with infected cells were washed twice with PBS solution and fixed with 2.5% glutaraldehyde/formaldehyde in PBS for 30 min. The samples were imaged with an atomic force microscope (Bruker Dimension Icon with ScanAsyst) in the Peak Force Quantitative Nanomechanical Mapping (QNM) imaging mode. A triangular silicon nitride cantilever with a 20 nm radius tip was used to scan at a speed of 0.2–0.5 Hz with resonant frequency of probe tip oscillations at 150 kHz.

**SEM analysis.** Coverslips with infected KC cells were washed twice with PBS. Cells were then fixed with 0.5% aqueous osmium tetroxide, followed by ethanol dehydration, hexamethyldisilazane treatment and carbon coating. Images were acquired with a Zeiss Ultra Plus FEG-SEM, operated at 1 kV and a working distance of 2 mm.

**Confocal laser-scanning microscopy.** Coverslips with infected Vero cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min and incubated in blocking solution (5% milk powder in PBS) for 30 min. Primary labelling was done with anti-β-tubulin antibody (Sigma) diluted 1:100 in blocking solution overnight at 4 °C, followed by three consecutive 5 min washes with 0.5% Tween 20 in PBS. Secondary labelling was with Alexa Fluor 594-conjugated anti-mouse antibody (Invitrogen) diluted 1:250 in blocking solution for 1 h at room temperature. After another set of washes, the coverslips were mounted onto microscope slides using VECTASHIELD mounting medium (Vector Laboratories) and analysed with a Zeiss LSM 510 Meta confocal laser-scanning microscope.

**High-pressure freezing and freeze substitution.** Infected cells cultured as a monolayer were dislodged by scraping, concentrated by low-speed centrifugation and the cell pellet transferred to a Leica membrane sample carrier. High-pressure freezing was done with a LEICA EMPACT2 high-pressure freezing apparatus followed by freeze substitution in a LEICA AF52 freeze substitution machine.

**Resin embedding and ultrathin sectioning.** Resin embedding was done in Quetol 651 epoxy resin (Van der Merwe & Coetzee, 1992) or LR White resin (SPI Supplies) for ultrastructural analyses and immunogold labelling, respectively. Ultrathin sections (100 nm) were
made with a Reichert-Jung Ultracut E microtome with a Diatome diamond knife.

**Immunogold labelling.** Immunogold labelling was done with a variety of AHSV-specific antibodies. Antibodies directed against NS2 or VP7, respectively, were raised in rabbits (Uitenweerde et al., 1995) or guinea pigs (Rutkowska et al., 2011) against these proteins expressed in insect cells by a baculovirus system. The rabbit anti-NS1 serum (GenScript) was directed against a synthetic peptide representing aa489–503 of NS1. Ultrathin cell sections on carbon grids were blocked in potassium phosphate buffer (PPB) containing 5% FCS and 0.05% Tween 20 for 30 min. Grids were transferred to a drop of primary antibody diluted 1:100 in blocking solution, incubated for 2 h at room temperature and washed twice consecutively for 2 min each in blocking solution, PPB and dH2O. Secondary labelling was done for 1 h with anti-rabbit or anti-guinea pig 10 nm colloidal gold conjugate (SPI Supplies) diluted 1:20 in blocking solution and the samples washed as above.

**Staining and TEM viewing.** Ultrathin sections from Quetol-embedded and immunogold-labelled LR White-embedded samples were stained for 15 min in 1% uranyl acetate and 3 min in Reynolds’ lead citrate (Reynolds, 1963). TEM visualization was done with a JEOL JEM-2100F field emission transmission electron microscope.

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