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Visualization of new virus-like-particles in *Trichomonas vaginalis*

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Abstract. In the present work, we demonstrate virus-like particles (VLPs) with various morphological variations in *Trichomonas vaginalis*. The VLPs were distinct based on size, shape and electron density, with VLPs being either electron-dense or electron-lucent. We used electron microscopy thin sections of several *T. vaginalis* strains virus-infected, and also negative staining of fractions obtained after purification by CsCl buoyant density gradient centrifugation. The particles observed in fractions are identical to those previously described, but by thin sections, we found new forms. The shapes found were icosahedral, spherical and oblong, and the sizes varied from 33 to 120 nm in diameter with the most common VLP being spherical and having a size range from 83 to 104 nm. The VLPs were found in the cytoplasm closely associated with the Golgi complex, with some VLPs budding from the Golgi, and other VLPs were detected adjacent to the plasma membrane. Unidentified cytoplasmic inclusions were observed in the region close to the VLPs and Golgi. Clusters of the already described icosahedral virus were also observed in the cytoplasm, although less frequently. These results indicate that *T. vaginalis* organisms may be infected with different dsRNA viruses simultaneously.

Keywords: *Trichomonas vaginalis*, dsRNA virus, virus-like-particles, ultrastructure

Introduction

Despite early knowledge of viruses in a wide range of organisms, viruses in protozoa were not identified until 1986. Early reports described virus-like particles (VLPs) in *Plasmodium*, *Naegleria*, *Leishmania*, and *Entamoeba* (Wang & Wang, 1991). Among the protozoan viruses, *Trichomonas vaginalis* virus is the first dsRNA virus identified to infect the sexually-transmitted pathogenic protozoan *T. vaginalis* (Wang & Wang, 1986). Numerous *T. vaginalis* clinical isolates are persistently infected with double-stranded RNA (dsRNA) viruses (Wang & Wang, 1986; Khoshnan & Alderete, 1993). The *T. vaginalis* virus presents dsRNA genome, and does not share significant sequence homology with other parasite virus, such as those from Giardia and Eimeria.

The replication of *T. vaginalis* dsRNA virus has been correlated with the surface expression and phenotypic variation of a highly immunogenic protein (Alderete et al., 1987). *In vivo*, trichomonads harboring the virus were found to exist as a heterogenous population of surface-expressing and non-surface-expressing trichomonads with respect to the immunogen and, without exception, only the virus-infected organisms switched between surface-plus and surface-minus phenotypes (Alderete et al., 1987).

Attempts to identify viruses in trichomonads by electron microscopy on thin sections, have not been successful. However, the virus has been observed by negative staining after purification (Wang & Wang, 1986; Wang et al., 1987; Khoshnan & Alderete, 1993; Bessarab et al., 2000). Only one paper has described the presence of clusters of particles resembling viral structures in the cytoplasm and also in the nuclei of one strain of *T. vaginalis* (Champney et al., 1995).

The trichomonads virus is described as a uniform population of icosahedral particles with a constant diameter of

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33 nm (Wang & Wang, 1986), but recently Bessarab et al. (2000) presented a distinct type of virus in *T. vaginalis*. This second virus-type is also a 33 nm particle.

Previous studies demonstrated that the virus-harboring trichomonads contain at least three unique dsRNA segments with sizes ranging from 4.8 to 4.3 kbp, associated with purified virus particles (Khoshnan & Alderete, 1993). These authors suggested the presence of a segmented virus or possibly infection of this protozoan by several different viruses. Other group (Tai et al., 1993), claimed for a divergence of *T. vaginalis* virus RNAs among various isolates. They found that the *T. vaginalis* viruses are related, but highly divergent. They also identified a subgenomic viral dsRNA fragments of about 0.5 kb in many isolates of infected *T. vaginalis*. Tai et al. (1995) found at least five different sizes of dsRNA in various *T. vaginalis* isolates, suggesting the coexistence of multiple dsRNA species due to coinfection by heterologous virus in the same cells.

While the significance of virus presence in the virulence and pathogenicity of *T. vaginalis* remains unknown, it is noteworthy the possibility of trichomonads serving as a vector of infectious agents. Finally, studies are necessary to identify the taxonomy of these viruses and the role, if any, they play in the overall biology of the parasite and the host–parasite interrelationship.

In the present study, we demonstrate VLPs in thin sections of *T. vaginalis* showing that they consist of a heterogeneous population and are often found closely associated with the Golgi complex and adjacent to the parasite surface membrane. Overall, these observations may indicate a greater complexity for viruses associated with *T. vaginalis* organisms than previously appreciated.

Materials and methods

Parasites

Trichomonads were routinely cultivated in complex Trypticase-yeast extract-maltose (TYM) medium supplemented with 5% heat-inactivated horse serum, as before (Alderete et al., 1987). Low iron medium was prepared by the addition to growth medium of 2,2-dipyridal (Sigma Chemical Co., St. Louis, MO) to a 150 mM final concentration (Lehker et al., 1991). High iron medium was made by the addition to growth medium of 250 mM final concentration ferrous ammonium sulfate-hexahydrate (Sigma) from a 100-fold stock solution made in 50 mM sulfosalicylic acid (Lehker & Alderete, 1992).

Trichomonas vaginalis isolate JT was isolated from a patient attending the Federal University Hospital in Rio de Janeiro, Brazil. *Trichomonas vaginalis* isolates NYH 286 is a long-term grown laboratory isolate while isolates T068-II and 347 are fresh clinical isolates, all of which have been used experimentally (Alderete et al., 1987; Khoshnan & Alderete, 1994a,b, 1995; Wang et al., 1987). Except for isolate JT, isolates T068-II, NYH 286 and 347 are comprised of trichomonads infected with the dsRNA virus, as described (Khoshnan & Alderete, 1994a,b, 1995; Wang et al., 1987). For some experiments, *T. vaginalis* 347 progeny organisms without virus and derived from the parental virus-infected parasites were used (Khoshnan & Alderete, 1994a; Wang et al., 1987). The virus-minus 347 trichomonads are referred to as 347v−.

Transmission electron microscopy (TEM) (routine preparation)

Cells were washed three times in phosphate buffer saline (PBS) at 37 °C and fixed overnight at room temperature (RT) in 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2); post-fixation was performed in 1% OsO_4 in cacodylate buffer plus 5 mM $CaCl_2$ and 0.8% potassium ferricyanide. Cells were washed, dehydrated in acetone, and embedded in Epon. Thin sections were stained and observed in a JEOL 1210 electron microscope.

Immunoelectron microscopy

Cells were washed three times in PBS and after overnight fixation (4% paraformaldehyde, 0.4% glutaraldehyde, 1% picric acid, 0.1 M cacodylate buffer, pH 7.2) samples were dehydrated in ethanol and then, infiltrated with Unicryl (BBInternational, Cardiff, UK). For immunolabeling thin sections were first incubated in PBS/albumin 3%, quenched in 50 mM NH_4Cl for 30 min, and incubated for 3 h in the presence of first antibody, the rabbit anti-capsid serum (Liu et al., 1998) was kindly provided by Dr Jung-Hsiang Tai of the Institute of Biomedical Sciences, Academia Sinica of Taipei, Taiwan. This antiserum was diluted 1:100 in PBS containing bovine serum albumin (BSA). Normal prebleed rabbit serum (NRS) was used as a negative control under the same experimental conditions. Subsequently, after several washes in PBS/albumin 1%, sections were incubated in the presence of 10 nm gold-labeled goat anti-mouse IgG (BBInternational). After several washes, sections were stained with uranyl acetate and lead citrate, and observed in a JEOL 1210 electron microscope. Controls were performed by omission of the primary antibody and immunolabeling in virus-minus isolates.

Lectin labeling

Cells were washed three times in PBS, and after overnight fixation in a solution of 4% paraformaldehyde, 0.4% glutaraldehyde, 1% picric acid, and 100 mM cacodylate buffer, pH 7.2, samples were dehydrated in ethanol prior to infusion in Unicryl (BBInternational). For labeling, thin sections were first incubated in a solution of PBS-3% albumin followed by quenching in 50 mM NH_4Cl for 30 min, and subsequently incubated for 3 h in the presence of concanavalin A (ConA), previously conjugated to 10 nm gold particles using Frens' method (1973). After several washings, sections were stained with uranyl acetate and lead citrate, and observed in JEOL 1210 electron microscope.

Controls for specificity

The specificity of the lectin-gold labeling was assessed, (a) incubation of the thin sections with 100 mM of the correspondent monosaccharide, prior the labeling; (b) incubation

of the thin sections with the unlabeled lectin followed by incubation with the gold complexed lectin.

Detection of dsRNA in virus-harboring isolate trichomonads

Screening and rapid detection of dsRNA in total nucleic acid preparations was performed as detailed before (Wang et al., 1987; Khoshnan & Alderete, 1993). Briefly, 2×10^7 of logarithmic-phase-growing trichomonads were pelleted and washed three times with PBS. Organisms were suspended in 500 ml PBS followed by addition of 25 ml 10% sodium dodecylsulfate (SDS). This lysate was mixed and twice extracted with an equal volume of unbuffered phenol at RT. Then, the aqueous phase was extracted twice with an equal volume of chloroform followed by mixing the aqueous phase with 50 ml of 3 M sodium acetate. To this was added 1 ml of cold 100% ethanol for incubation at −20 °C for 30 min, and precipitated nucleic acids were pelleted by centrifugation. The pellet was washed an additional time with ethanol before drying. Dried nucleic acids were dissolved in 20 ml of diethylpyrocarbonate-treated distilled water. An amount of 10 ml was analyzed by electrophoresis using 1% agarose, and the presence or absence of the dsRNA was determined by ethidium bromide staining. The dsRNA was also purified as before (Khoshnan & Alderete, 1993) from selected isolates to reaffirm the relationship between the virus and the dsRNA detected in agarose gels. As shown previously, the absence of dsRNA among trichomonads of some isolates gave no detectable virus particles following purification under the exact experimental conditions as those performed for the virus-harboring organisms. All experiments were performed no less than three times using duplicate samples.

Detection of virus capsid protein by immunoblot

Standard SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gels and immunoblotting of trichloroacetic acid-precipitated total proteins of *T. vaginalis* organisms was performed using protocols detailed before (Alderete et al., 1987; Khoshnan & Alderete, 1994a; Laemmli, 1970). For immuno-detection of capsid protein, rabbit anti-capsid serum (Liu et al., 1998) was kindly provided by Dr Jung-Hsiang Tai of the Institute of Biomedical Sciences, Academia Sinica of Taipei, Taiwan. This antiserum was diluted 1:1000 in PBS containing skim milk. Normal prebleed rabbit serum (NRS) was used as a negative control under the same experimental conditions. NRS did not react with any trichomonad proteins.

Virus particle purification

Approximately 4×10^9 trichomonads were suspended in TNM buffer (150 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris, pH 7.5) and subjected to sonication until more than 90% of the cells were lysed. The lysate was clarified by centrifugation at least twice at 10 000 *g* for 20 min in a Sorvall SS34 rotor. The supernatant was then pelleted through a 20% sucrose cushion prepared in TNM buffer at 100 000 *g* in a SW40 rotor for 2 h. The sediment containing the virus particles was suspended in TNM buffer, equilibrated to a density of 1.35 g/ml with CsCl, and re-centrifuged at 10 000 *g* for 24 h. Twenty-four fractions of 0.5 ml each were collected from the bottom of each tube and extensively dialyzed in TNM buffer. The fraction with the lightest density was designated fraction 1. One milliliter of each fraction was then treated with 50 g/ml proteinase K and 1% SDS at 65 °C for 30 min before phenol–chloroform extraction. The RNA was precipitated with three volumes of ethanol and separated in a 1% agarose gel as described above. Fractions containing the viral dsRNA were further examined for the presence of virus particles by negative staining with 5% uranyl acetate and observed in a JEOL 1210 electron microscope.

Results

Figure 1A shows the presence of dsRNA among trichomonads of known virus-infected *T. vaginalis* isolates, such as isolates NYH 286 (lane 1), 347 (lane 3), and T068-II (lane 4) (Khoshnan & Alderete, 1993, 1994a; Wang et al., 1987). The intensity of EtBr-stained dsRNA was similar in the lanes loaded with identical cell equivalents (lanes 1, 3 and 4). Likewise, no dsRNA was detectable for virus-minus progeny organisms derived from the virus-infected parental 347 isolate (lane 2) as well as for isolate T016 trichomonads without virus (lane 5) (Khoshnan & Alderete, 1994a). The dsRNA derived from CsCl-purified virus (Khoshnan & Alderete, 1994a; Wang et al., 1987) gave bands that migrated identically to the dsRNA in total nucleic acid preparations (Fig. 1A), and no virus was ever detected within the virus-minus organisms.

That the viruses within these distinct NYH 286, 347, and T068-II isolates were dissimilar is shown in Figure 1B. Specific anti-capsid serum (Liu et al., 1998) recognized only virus from isolates NYH 286 and 347, but not T068-II. Interestingly, although the intensity of dsRNA was relatively similar in identical cell equivalents (Fig. 1A, lanes 1, 3 and 4), the intensity of antibody binding was stronger for isolate NYH 286 than 347 (Fig. 1B). This indicated either greater numbers of viral particles within NYH 286 or, as suggested by others for some virus-infected organisms (Liu et al., 1998), the possibility of multiple viruses within 347, only some of which were recognized by the antisera. Figure 1C presents the stained proteins patterns showing the loading of equivalent amounts of proteins in each lane.

Virus-harboring trichomonads have a diversity of VLPs. Examination of thin sections of trichomonads of *T. vaginalis* isolate JT revealed an ultrastructure in which the Golgi, cytoplasm, the hydrogenosomes, an organelle involved in pyruvate metabolism showed routine distribution and normal electron density (Fig. 2), as previously described by us and others (Honigberg & Brugerolle, 1990; Furtado & Benchimol, 1998). Similar observations were made upon examination of the virus-minus progeny 347v− derived from isolate 347.

However, examination under identical conditions of thin sections of virus-harboring trichomonads revealed an overall different morphology. We readily observed dense structures

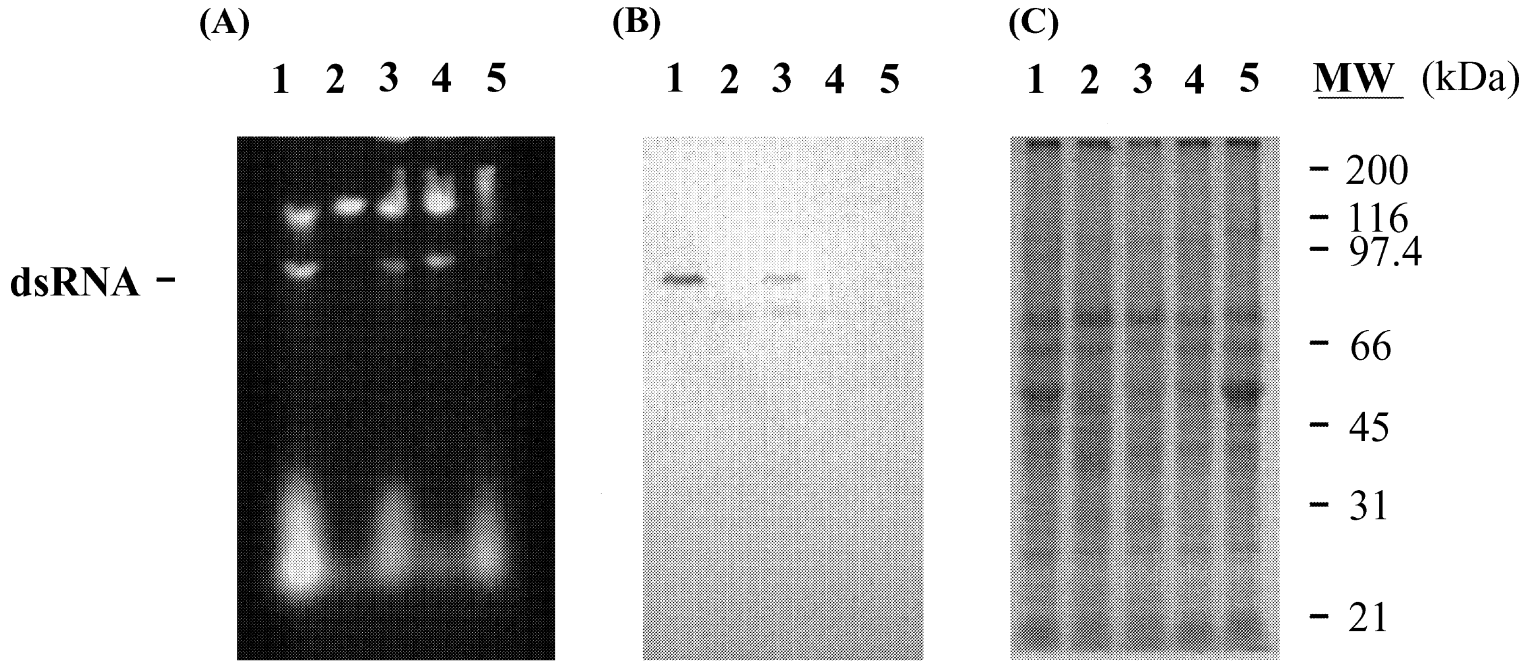
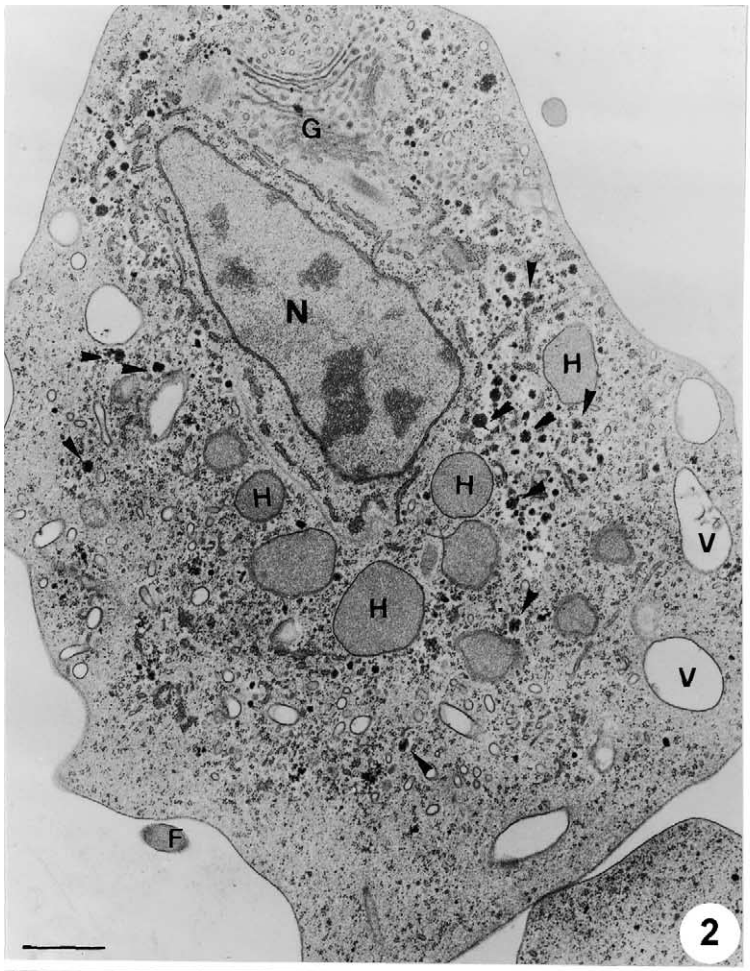


Fig. 1 Demonstration of virus dsRNA and immunoblot detection of virus-capsid protein in virus-harboring trichomonads. Nucleic acids and total proteins of isolates NYH 286 (lane 1), virus-minus progeny 347v– (lane 2), 347 parental (lane 3), T068-II (lane 4) and T016 (lane 5) were examined for the presence of dsRNA (A) and capsid protein using rabbit anti-capsid serum (B) as described in Section 'Materials and methods'. For part A, note the absence of dsRNA from the 347v– progeny (lane 2) and the virus-minus isolate T016 (lane 5) compared to NYH 286, T068-II, and the parental, virus-infected 347 (lane 3), as before (Khoshnan & Alderete, 1994a). Bands were visualized by staining with ethidium bromide. The top band represents trichomonal DNA. For part B, rabbit anti-capsid serum is immuno-crossreactive with capsid protein of isolates NYH 286 (lane 1) and 347 (lane 3) but not T068-II (lane 4), showing that T068-II is infected with a distinct dsRNA virus (Liu et al., 1998). This absence of immunoreactivity with T068-II virus capsid served as an internal control. Further, NRS used as a negative control was unreactive with any proteins in the total TCA protein preparations of lanes 1–5). The stained protein patterns in part C are presented to show the loading of equivalent amounts of protein in each lane. MW refers to the molecular weight of marker proteins in kilodaltons (kDa).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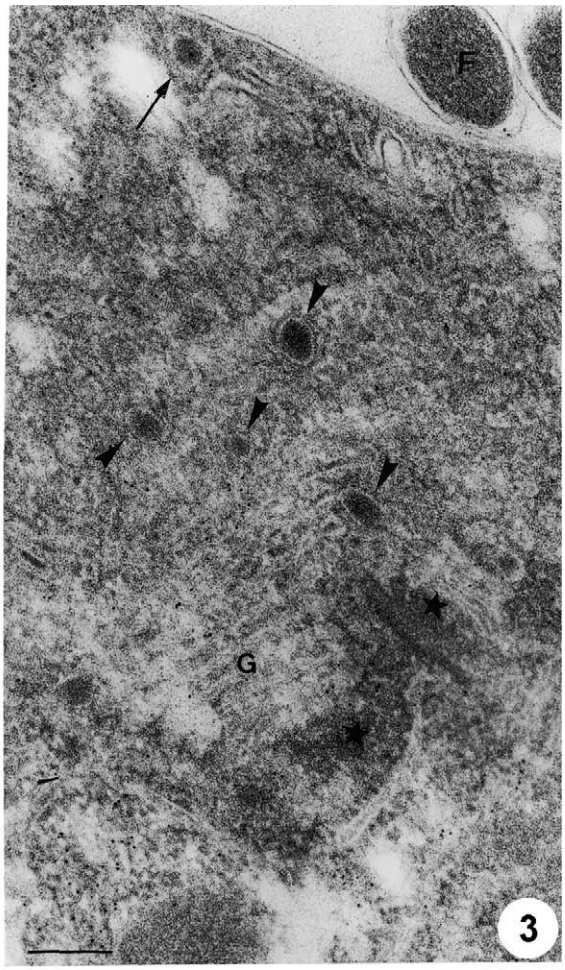
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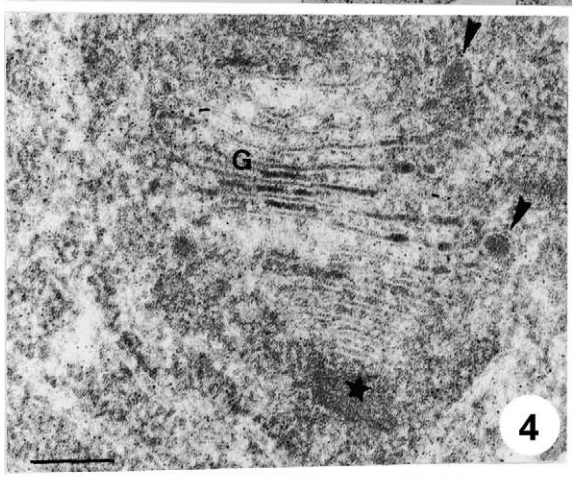
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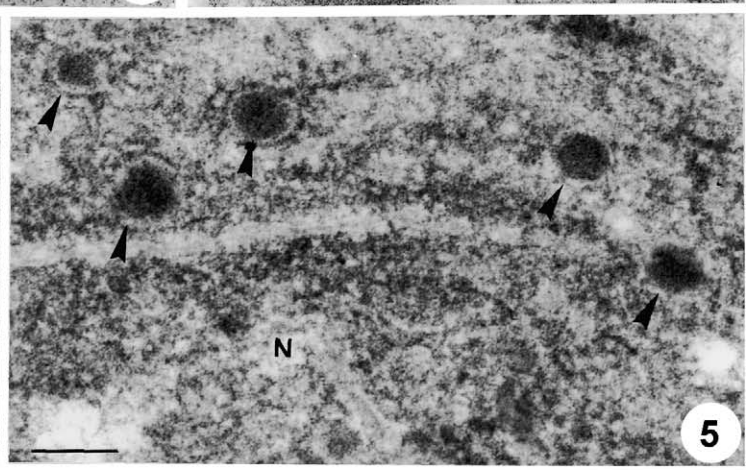
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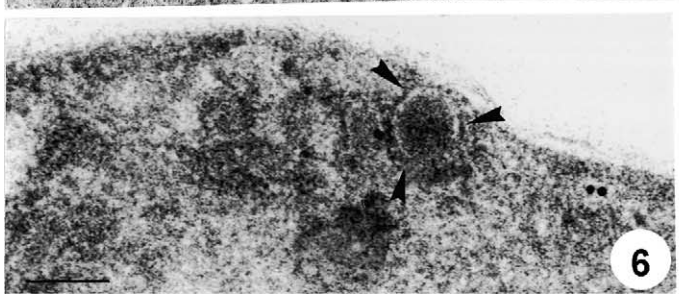
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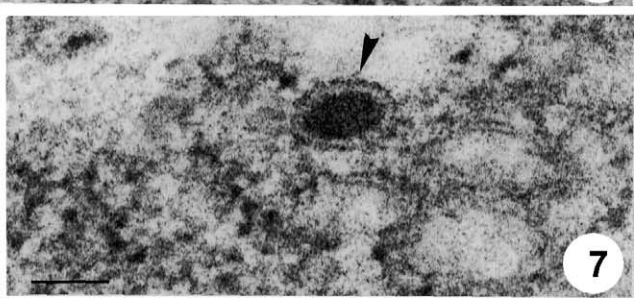
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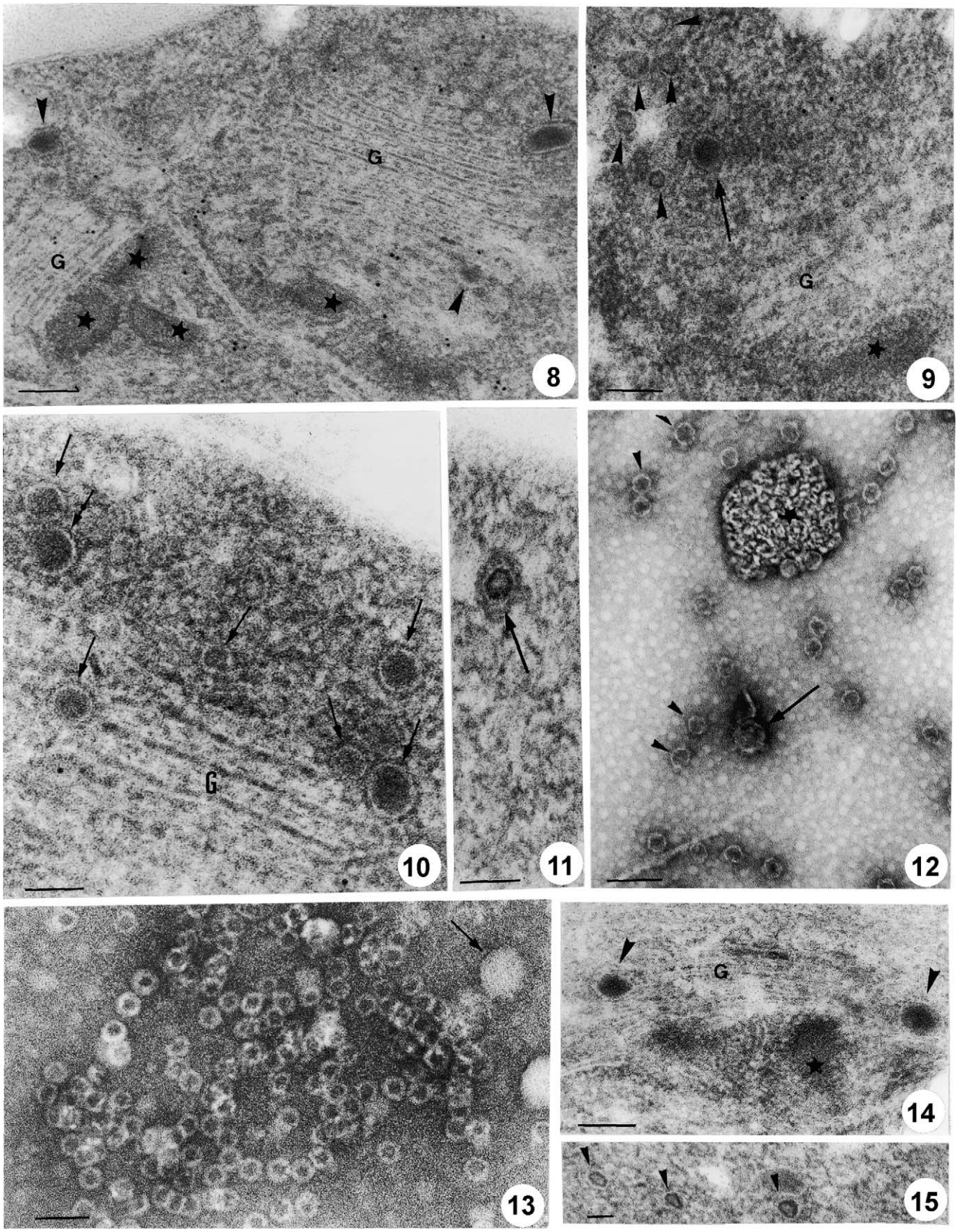
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Fig. 2 General view of a virus-free *T. vaginalis*, isolate JT, in a routine thin section showing the overall organelles distribution, hydrogenosomes (H), nucleus (N), vacuoles (V). Note that no VLPs are observed, the Golgi (G) does not present dense bodies, and the dense particles distributed over the cytoplasm are glycogen granules (arrowheads). F, flagellum. ×15 000. Bar = 650 nm. **Fig. 3** An infected cell, strain 347+, grown in low iron medium, labeled with rabbit anti-capsid serum shows labeling over the Golgi (G), and cytoplasm, but not in the VLPs (arrowheads). Cytoplasmic inclusions (stars) are seen in close proximity with the Golgi presenting VLPs (arrowheads) scattered in the cytoplasm. These particles present sizes varying between 56 and 84 nm. Notice that one of the particles is adjacent to the plasma membrane (arrow). F, flagellum. ×72 000. Bar = 140 nm. **Fig. 4** An infected cell, strain 347+, grown in low iron medium, showing an uncommon density in the Golgi complex (G). At rims of this structure, VLPs measuring 46–70 nm are seen (arrowheads). A cytoplasmic dense inclusion is also depicted (star). ×64 000. Bar = 150 nm. **Fig. 5** A high magnification of perinuclear region from an infected-cell, strain 347+, grown in low iron medium. VLPs (arrowheads) with diameter of 94–104 nm and presenting a dense core, an outer coat and a middle electron-lucent layer are seen. N, nucleus. ×96 000. Bar = 100 nm. **Fig. 6** A VLP (arrowheads) measuring 104 nm diameter is seen adjacent to the plasma membrane of an infected cell, strain 347+, grown in low iron medium. ×96 000. Bar = 100 nm. **Fig. 7** An oblong-shaped VLP (arrowhead) measuring 75 nm × 125 nm is seen in a strain 347+, grown in low iron medium. It presents an outer electron-dense coat, a middle electron-lucent layer and a dense solid core. ×120 000. Bar = 80 nm.

Fig. 8 An infected-cell, NYH isolate, labeled with rabbit anti-capsid serum shows labeling over the cytoplasm, but not in the VLPs (arrowheads), which measure 69–138 nm, and some are oblong-shaped. Cytoplasmic inclusions (stars) are seen in close proximity with the Golgi (G). ×72 000. Bar = 140 nm. **Fig. 9** An infected cell, strain 347+, grown in low iron medium, showing VLPs with various morphological variations (arrowheads), measuring between 83 and 111 nm. Some appear empty, with hollow or incomplete cores. The arrow points to a VLP that seems assembled, with a dense core. ×72 000. Bar = 140 nm. **Fig. 10** *Trichomonas vaginalis*, isolate 347+. Several VLPs (arrows), with sizes varying between 70 and 90 nm, are found in close association with the Golgi complex (G). They present a dense core, a middle electron-lucent layer and an outer coat. Notice that some particles are close to the cell surface. ×112 000. Bar = 90 nm. **Fig. 11** *Trichomonas vaginalis*, isolate T068-II. An icosahedral VLP measuring 50 nm, presenting a dense core is seen (arrow). ×96 000. Bar = 100 nm. **Figs 12** & **13** Electron micrographs of dsRNA-containing samples from fractions of *T. vaginalis*, isolate 347+, submitted to the CsCl buoyant density gradient centrifugation, and negatively stained with uranyl acetate. **Fig. 12** A cluster of particles is seen (star) close to VLPs of 33 and 54 nm. ×112 000. Bar = 90 nm. **Fig. 13** The population is formed by particles of 33 nm, with few of 104 nm (arrow). ×96 000. Bar = 100 nm. **Fig. 14** An infected cell, strain 347+, grown in low iron medium, showing VLPs with 125–140 nm, in close association with the Golgi complex (G). A cytoplasmic inclusion (star) is seen in close proximity with the Golgi. ×55 000. Bar = 170 nm. **Fig. 15** An infected cell, strain 347+, grown in low iron medium, showing VLPs with 44–50 nm. ×80 000. Bar = 60 nm.

reminiscent of VLPs of variable size (33–120 nm) and with spherical and oblong shapes. The small (33 nm) and larger particles (55–120 nm) did not present labeling when the anti-capsid antibody was used in immunolabeling electron-microscopy (Fig. 8). They were found scattered over the cytoplasm, with a preferential location close to Golgi complex. Observations were made indicative of VLP budding (Figs 3, 4, 8, 10 & 14). VLPs were observed in the cytoplasm (Figs 8–11 & 15) and close to the plasma membrane, indicating the possibility for VLP trafficking (Figs 3 & 6). Of interest was the presence within virus-infected trichomonads of electron dense VLPs juxtaposed to nucleus (Fig. 5), including unidentified cytoplasmic inclusions in the surrounding region (Figs 3, 4, 8, 9 & 14). Importantly, similar structures were not observed in virus-free cell samples (Fig. 2). Because of the frequent association of these dense cytoplasmic inclusions in cells virus-infected, we regard them as "viral footprints".

ConA binding sites were seen on luminal faces of the vacuoles, Golgi and ER membranes, but no labeling was found on the VLPs (not shown).

We have been able to detect the VLPs both in thin sections (Figs 3–11, 14 & 15) and also in isolated fractions obtained from *T. vaginalis* and seen by negative staining electron microscopy (Figs 12 & 13). Observation of all isolates fractions by negative staining showed an almost uniform spherical VLP with an estimated diameter of 33 nm (Figs 12 & 13).

We were able to detect by thin sections electron microscopy the different sizes and shapes of VLPs within the virus-infected *T. vaginalis* isolates 347, T068-II, and NYH 286. Isolate 347 had an especially pronounced concentration of VLPs readily detectable (Figs 3–6 & 10) when compared to the other virus-harboring isolates (Figs 8 & 11). Within isolate 347, VLPs were not uniform, and various morphologies were apparent (Figs 3–7, 9, 10 & 14). Interestingly, the VLPs had different electron densities as shown in Figures 9 and 10, and VLPs were not found in the nucleus. The sizes of VLPs included (1) <40 nm (Figs 12 & 13), (2) between 44 and 70 nm (Figs 4, 11, 12 & 15), (3) 70 and 80 nm (Figs 3 & 10), (4) 83 nm (Fig. 9), (5) 92 nm (Fig. 10), (6) 100 and 104 nm (Fig. 9), (7) >110 nm (Figs 6 & 14). The most commonly seen size of VLPs was 83 nm.

Analysis of the different shapes of the VLPs indicated the following: (1) the smallest VLP of ~30 nm was icosahedrical (Figs 8 & 15), (2) both spherical and oblong VLPs had a dense core, a middle intermediate electron-lucent region, and an outer coat (Figs 3–10), (3) spherical VLPs in the range of 83–104 nm (Figs 3, 5 & 6). This latter group of VLPs were the most frequent observed within trichomonads. In very few occasions we found crystalline structures.

The core of the VLPs had different electron densities (Fig. 9) and sizes (Figs 9 & 10) when visualized within thin sections of the same preparation. VLPs were detected either with a solid or hollow core (Figs 5, 9 & 10). Importantly, the different electron densities do not depend upon the type of fixation that was employed, as evidenced by the fact that the same cell had VLPs of different electron densities (Figs 5, 9 & 10).

Relationship between extent and number of VLPs and stressful growth conditions

During the course of our investigation, we decided to examine trichomonads grown in iron-replete and iron-limiting media, as iron is known to influence the expression of important properties and proteins (Alderete, 1999; Lehker & Alderete, 1992). The extent and number of the different VLPs was

dramatically increased when organisms were grown in low iron medium. It was readily apparent that numerous changes appeared within the thin sections derived from virus-infected *T. vaginalis* parasites. For example, dense inclusions were visualized in the cytoplasm. In addition, parallel Golgi cisternae with dense material was detected, and the electron dense VLPs had a large variation in morphology (Figs 3, 4, 8, 9 & 14). Thin sections of trichomonads grown in high iron medium were similar to normal-grown parasites and did not have the increased density of the different VLPs as seen for low iron grown organisms (data not shown). However, the VLPs were most observed on low iron grown organisms (Figs 3–10).

Infected *T. vaginalis* cultures of isolates 347, NYH 286 and T068-II were fractionated and viruses were pelleted from the homogenates and further purified in CsCl buoyant density-gradient centrifugations. The purified sample contains the double-stranded RNA (Fig. 1). Observation of the fractions in the electron microscopy by negative staining revealed the presence of icosahedral virus-particles, mostly of 33 nm diameter. Some larger particles were occasionally found (Figs 12 & 13).

Discussion

A linear dsRNA has been identified in many isolates of the parasitic protozoan *T. vaginalis* (Wang & Wang, 1986). The dsRNA virus was pelleted with membranes from trichomonad homogenates, which was further purified using standard in CsCl centrifugation to give dsRNA virus with a major capsid protein of ~85 kDa. Negative staining electron microscopy revealed icosahedral virus of 33 nm diameter. No virus was detectable by thin sections examined by transmission electron microscopy (Wang & Wang, 1986). More recently, virus particles were visualized in the nucleus and cytoplasm and between the nucleus and the axostyle (Champney et al., 1995). Three distinct dsRNAs were found associated with virus-harboring trichomonads (Khoshnan & Alderete, 1993), suggesting the coexistence of these dsRNA species would reflect the presence of multiple viral infections or the presence of a single tripartite genome for the dsRNA virus. Interestingly, studies have shown the existence of at least five dsRNA species among some *T. vaginalis* isolates (Tai & Ip, 1995; Su & Tai, 1996). Not surprisingly, therefore, two viral capsid proteins have been detected in a particular isolate of *T. vaginalis* (T1), each associated with a distinct type of virus (Bessarab et al., 2000). Electron microscopy examination of virus by negative staining led these authors to the observation of spherical-shaped virions of 33 nm. It is noteworthy that all previous reports showed icosahedral dsRNA virus with 33–40 nm diameters.

In the present work, we demonstrate the trichomonads possess VLPs with a wide size range and with different shapes. We also were able to examine these VLPs *in situ*, in thin sections of Unicryl embedded *T. vaginalis*. The VLPs location was cytoplasmic and frequently detectable in the Golgi area. In virus-minus trichomonads, such as isolate JT or of the virus-minus 347 progeny parasites, the Golgi complex consists of 8–12 flattened cisternae that often display an electron-lucent contents. In virus-infected parasites, the Golgi present some distinct structural modifications, expressed in a variety of parameters including (a) wider Golgi cisternae, (b) larger intercisternal spacing, (c) thicker and more electron-dense cisternal contents, (d) presence of intercisternal elements, and (e) presence of different-sized secretory vesicles with varied contents. The electron dense material of the Golgi was found very similar to the density of VLPs. It was frequently observed on the Golgi dilated rims and even budding from them. On the other hand, this Golgi material was not found previously in virus-minus organisms. It is conceivable that, if the VLPs represent *bona fide* virus, a virus-assembly pathway occurs through the Golgi complex, probably for addition and/or modification of carbohydrates, as demonstrated in the morphogenesis of other viruses (Green et al., 1981; Griffiths et al., 1982). However, ConA-gold conjugate, which recognizes α-D-mannose and α-D-glucose residues, did not show reaction on the VLPs, although the membranes of Golgi, ER, and vacuoles were intensely labeled (not shown). The presence of other sugars are under investigation. After immunolabeling with anti-rabbit anti-capsid antibody the VLPs did not present any positive reaction, reinforcing that these particles are different to the previously 33 nm virus already described (Wang & Wang, 1986). As previous work showed the dsRNA virus closely associated with membrane fractions of virus-infected trichomonads (Wang et al., 1987), it is possible that the preparations contained membrane fractions from the Golgi complex. That some VLPs were also observed in close proximity to the plasma membrane may indicate a route for translocation of the VLPs to the plasma membrane. Studies are necessary to test for this hypothesis.

Several described virus-infected cells present cytoplasmic inclusions. They are seen as structures of variable morphology (Ghadially, 1997). Almost all virus-infected *T. vaginalis* organisms had dense cytoplasmic inclusions that were variable in number and morphology. Of interest is that these inclusions were always in the Golgi region. It is noteworthy that similar inclusions have been observed in higher eukaryotic cells infected with viruses (Ghadially, 1997). Therefore, it is reasonable to hypothesize that the inclusions detected within trichomonads may also be of viral origin.

Until today only Champney et al. (1995) were able to see the *T. vaginalis* virus in thin sections but they were isometric particles of 30–40 nm in diameter. In the other studies, the authors claimed that even after a careful screening they were not able to find the virus particles under electron microscopy (Wang & Wang, 1986). The difficulty in identifying viruses in *T. vaginalis* under electron microscope could be due to the relatively small number of these viruses within trichomonads, estimated to be between 280 and 1380 particles per cell (Wang & Wang, 1985). We have two possible explanations to account for the absence in visualization of the virus(es). The

33 nm virus within purified fractions were of low electron density, requiring the aggregation of these small viruses in clusters to be recognized by the observer. On the other hand, for electron microscopy, every cell is cut in about 100 sections, and the transparent virus particle may be almost invisible within the thin sections. Furthermore, it may be difficult to detect virus present in low copy number (Wang et al., 1987). We found that the viruses are better visualized under stressful iron-limiting growth conditions. Indeed, it has been shown that steady-state growth under stressful nutrient-limiting conditions resulted in multiplication and release of dsRNA virus from the *T. vaginalis* isolate 347 trichomonads (Provenzano & Alderete, 1997), reinforcing the observation of increased virus production under certain conditions. Additionally, the fresh serum used in the TYM may contain antibodies or other potentially inhibitory factors (i.e. lytic enzymes) which could limit virus survival or release (Champney et al., 1995). It is equally conceivable that a lack of knowledge regarding the assembly cycle of the virus would make it difficult for investigators to know where to more closely examine for virus presence.

We found some of these VLPs morphologically similar to a DNA virus similar to herpes simplex virus (HSV) described in some monkey kidney cells (Ghadially, 1997). In this case, the HSV and ~103 nm VLPs have a similar morphology. However, contrary to the VLPs of *T. vaginalis*, the herpes viruses are found in the intranuclear region. It is noteworthy, however, that *T. vaginalis* inoculated genital HSV (Pindak et al., 1989) were able to internalize the virus into vacuoles followed by elimination of the virus. Our work shows that a better purification of the distinct VLPs is needed to more fully characterize the nature of these potentially novel viruses within trichomonads.

While the significance of virus presence in the virulence and pathogenicity of *T. vaginalis* remains unknown, it is noteworthy that the possibility of trichomonads serving as a vector of infectious agents. Finally, studies are necessary to identify the taxonomy of these viruses and the role, if any, they play in the overall biology of the parasite and the host–parasite interrelationship.

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