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Multinucleate host cells induced by *Vittaforma corneae* (Microsporidia)

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Abstract. The microsporidium *Vittaforma corneae* (Shadduck, Meccoli, Davis et Font, 1990) develops within the target cell cytoplasm. In the present study, green monkey kidney (E6) cells infected at 30°C, 35°C or 37°C with *V. corneae* developed enlarged multinucleate structures of up to 200 μm in any horizontal dimension made up either of a single cell or of multiple fused cells. A number of epithelial cell types (SW-480, HT-29, Caco-2 and HCT-8) were infected with *V. corneae* but did not induce the same highly organized structures, suggesting that for the structure to develop, the host cell must be capable of continued mitosis, and not be differentiated or be detaching from the surface matrix. Live cell imaging of infected E6 cells revealed large, multinucleate infected cells characterized by a central focus from which radiated parasite stages and host cell mitochondria. Immunocytochemistry identifying γ and α tubulin suggested that a single centrally-located microtubule organizing centre governed the distribution of parasite stages and host cell organelles, with mitochondria and parasites being eventually transported towards the periphery of the structure. Whole cell patch clamp analysis of infected cells indicated an average five-fold increase in total membrane capacitance, consistent with an enlarged single cell. Scanning electron microscopy revealed cell-like protrusions around the periphery of the structure with the intervening space being made up of parasites and cell debris. Clearly in the case of *V. corneae*-infected E6 cells the parasite-host cell relationship involves subverting the host cell cytoskeleton and cell volume control, providing the parasite with the same protected niche as does a xenoma.

Microsporidia are ubiquitous intracellular parasites, originally thought of as protozoa and now considered closely related to fungi (van de Peer et al. 2000). With well over 1,000 species of microsporidia infecting species in every major animal phylum, it is not surprising that many divergent host-parasite relationships have evolved. As obligate intracellular parasites the microsporidia appear to have been able to reduce their genome size, simplify their organelle structures and reduce their biochemical pathways by subverting the host cell's physiology to provide the missing biochemical support for parasite development (Slamovits et al. 2004). As with other intracellular parasites, from viruses to protozoa (e.g., Halonen and Weidner 1994, Risco et al. 2002, Grieshaber et al. 2003), the microsporidia appear to modify host cell cytoskeleton, organelle arrangement, biochemistry and cell cycle to optimize the intracellular niche (Weidner et al. 1999, Scanlon et al. 2000, 2004). To date, compared with many other infectious agents, relatively little is known about how the microsporidia signal the host cells to affect these many changes that occur in host cell structure and function.

A xenoma is perhaps the most dramatic example of the extent of a microsporidia parasite's control over host cell structure and function (Lom and Nilsen 2003). In several species of hosts, microsporidia cause gross enlargement of the host cell. The single host nucleus becomes distorted while virtually all stages of the parasite can be observed developing free of identification by the host's immune system. Xenomas may get as large as several millimetres while still remaining a single cell with one, albeit significantly modified, nucleus. It is possible that the host cell nucleus divides with some microsporidia species (e.g. Faye et al. 1991). In their early stages xenomas provide the parasite with an environment free of host immune surveillance (Dyková et al. 1980). The present study was undertaken to describe the development of what was initially believed to be a xenoma-like structure seen in cultured green monkey (E6) cells infected with *Vittaforma corneae* (Shadduck, Meccoli, Davis et Font, 1990) (Davis et al. 1990, Shadduck et al. 1990, Silveira and Canning, 1995). Unlike a true xenoma, however, these structures are multinucleate with a pronounced central microtubule organizing centre (MTOC), and they lack the surrounding fibroblasts and other supporting structures usually seen *in vivo*.

MATERIALS AND METHODS

Parasite cultures. *Vittaforma corneae* (ATCC 50505) was carried in continuous culture in green monkey (Vero, E6)

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kidney cells (ATCC CRL-1586) maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 10 µg/ml gentamicin and 0.5 µg/ml amphotericin B (Visvesvara et al. 1999). Cultures were maintained in 5% CO_2-air at 35°C.

Infection of cultured cells. The following cell lines were cultured in the media that were recommended by the American Type Culture Collection, SW-480 (ATCC CCL-228), HT-29 (ATCC HTB 38), HCT-8 (ATCC CCL 244) and Caco-2 (ATCC HTB-37). With the exception of the SW-480 cell line, all cultures were maintained in 5% CO_2-air at 35°C immediately before and after infection with purified *V. corneae* spores. The SW-480 cells were cultured in Liebovitz medium, supplemented with 10% foetal bovine serum, free of antibiotics, maintained in air at 35°C. Two additional sets of experiments were performed, one at 30°C, and one at 37°C. Essentially the same structures were observed in cells cultured at all three temperatures. However, because the multinucleate infected cells described here were optimal at the two lower temperatures while cell growth was optimal at the two higher temperatures, only the cultures grown at 35°C are described in detail in the present communication.

Immunocytochemistry. In one experiment the distribution of intracellular parasite stages and host cell mitochondria was determined in viable *V. corneae*-infected E6 cells using the method described previously (Leitch et al. 1997, Scanlon et al. 2004). Briefly, host cells were loaded with the fluorescent probe, calcein, which in the host cell is found as an impermeable free acid incapable of passing the parasite plasma membrane, and MitoTracker-CMXRos which labels mitochondria. This method allows visualisation of the relationship of host cell mitochondria to negatively stained parasite stages, using confocal microscopy.

Infected and uninfected cells were cultured in 4- and 8-well chamber slides for immunofluorescent microscopy. Cells were fixed with methanol at –20°C for 15 min, rinsed 3 times with Tris buffered saline (TBS), then blocked with 2% bovine serum albumin (BSA) in TBS for 10 min. In one group of experiments, cultures were double labeled for α tubulin and parasite stages, and in another group of experiments, for γ tubulin and parasite stages. The primary antibodies used were mouse anti-α tubulin (Clone B-5-1-2, Sigma Chemical Co., St. Louis, MO) diluted 1:500 or mouse anti-γ tubulin (Clone GTU-88, Sigma Chemical Co.,) diluted 1:500, together with rabbit anti-*V. corneae* (CDC), diluted 1:500 in 1% BSA in TBS. Samples were incubated for 1 h at 37°C. After 3 rinses in TBS the secondary antibodies were applied. These were goat anti-mouse IgG-Biotin (Jackson ImmunoResearch, West Grove, PA) diluted 1:300, together with goat anti-rabbit-AlexaFluor 594 (Molecular Probes, Eugene, OR) diluted 1:750 in 1% BSA in TBS. Samples were incubated for 1 h at 37°C, then washed 3 times in TBS before exposure to Streptavidin-Oregon Green 488 (Molecular Probes), diluted 1:300, for 45 min at room temperature. In some experiments nuclei were visualised by labeling samples with DAPI, diluted 1:1,000, for 5 min. Samples were then washed 3 times in TBS and mounted for fluorescent or confocal microscopic visualisation.

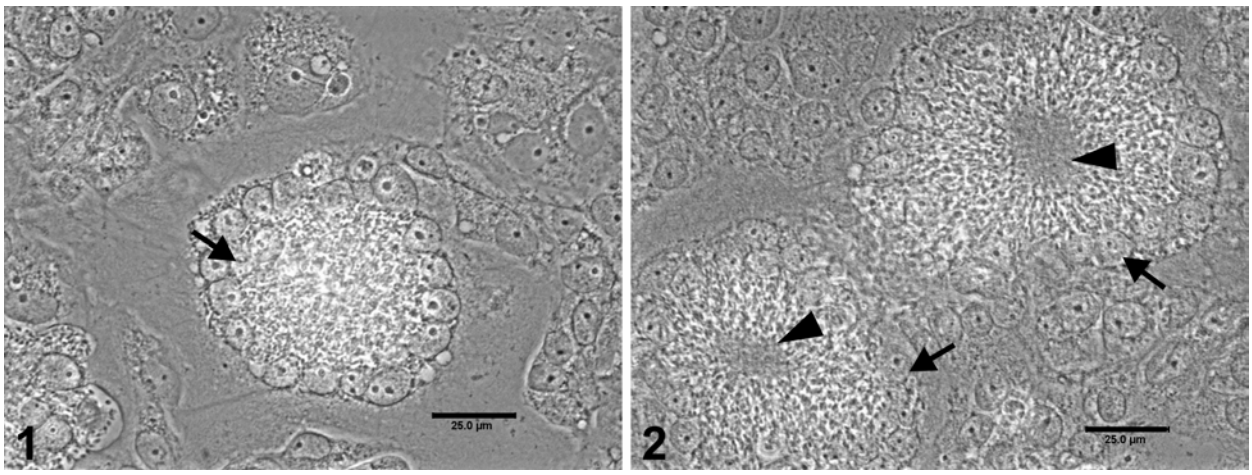
Cell capacitance measurements. To measure cell capacitance, as a reflection of cell size, in uninfected or infected E6 epithelial cells, we used the patch-clamp technique (Hamill et al. 1981) with an Axopatch 200A amplifier (Axon Instruments) and 4 M borosilicate patch pipettes to gain access to the intracellular milieu using the whole-cell configuration. Under our experimental conditions we used a pipette solution containing (in mM): 150 KCl, 0.1 EGTA, 1 $CaCl_2$ (free $[Ca^{2+}]$ = 26.5 nM) and 10 HEPES, pH to 7.2 with KOH and a standard bath solution containing (in mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, and 10 HEPES, pH to 7.3 with NaOH (Colden-Stanfield 2002). All experiments were performed at 22–24°C after correction of junction potentials. Total membrane capacitance (C_m) was measured in the whole cell mode by integrating the capacity transient. C_m was compared among the uninfected and infected groups using the unpaired Student's *t*-test.

RESULTS

The data presented here were obtained when E6 cells were infected with *V. corneae* and the cultures were carried at 35°C. Figs. 1 and 2, respectively, show confocal bright field images of an early and later stages of multinucleate cells seen when E6 cells were infected with *V. corneae*. A similar appearance was seen when the infected cells were cultured at 30°C or 37°C, although the appearance of the cells was more organized and uniform at the lower temperature and less organized at the higher temperature. Typically the infected cell had a central focus with material, including parasite stages, radiating outwards. Multiple host cell nuclei were observed near the periphery of the cell. With time, the central region of the cell collapsed leaving a space, as shown in both multinucleate cells in Fig. 2.

A total of five epithelial cell lines were infected with *V. corneae*. These were the undifferentiated E6 and SW-480 cells, the poorly differentiated HT-29 cells and the highly polarized and differentiated Caco-2 and HCT-8 cells. HT-29 was poorly differentiated under the culture conditions used in the present study. Table 1 summarizes some of the properties of these cell lines. With the exception of the E6 cells these cell lines are all derived from human colonic carcinomas. Only the E6 cell gave rise to the large, highly organized, multinucleate infected cells described here. SW-480 cells are often multinucleate so that one would have predicted that the structure described here in infected E6 cells would have also occurred in the SW-480 cells. However, large multinucleate SW-480 cells were only very occasionally observed and these were seen with the same frequency in both infected and uninfected cultures and did not have the same level of organisation nor the large size seen in the infected E6 cells. Comparing the properties of the various cell lines, one would conclude from Table 1 that the organized multinucleate infected cell configuration was favoured in undifferentiated host cells that remained attached to the culture flask for an extended period and resisted apoptosis/necrosis.

We previously described an assay that could be used in viable microsporidia-infected cells in which calcein



Figs. 1, 2. Confocal bright field images of *Vittaforma corneae*-infected multinucleate E6 cells. **Fig. 1.** Multinucleate cell (arrow), showing the peripheral location of the host cell nuclei. **Fig. 2.** Two multinucleate cells (arrows) showing collapsed centres to the structures (arrowheads). Scale bars = 25 µm.

Table 1. Summary of properties of cell lines tested for the development of highly structured multinucleate cells when infected with *Vittaforma corneae.*

Property	E6	SW-480	HT-29	Caco-2	HCT-8
V. corneae-induced multinucleate cell	yes	no	no	no	no
Differentiated, polarized monolayer	no	no	poorly	yes	yes
Uninfected cells remain attached, little apoptosis	yes	moderate	moderate	yes	yes
Mononucleate/multinucleate uninfected cell	mononucleate	both	mononucleate	mononucleate	mononucleate

was employed to negatively stain parasite stages and in which MitoTracker was used to probe host cell mitochondria (Scanlon et al. 2004). Fig. 3 illustrates the application of this method to study the *V. corneae*-induced multinucleate structure. In this and other images there appears to be a central focus, from which parasite stages and mitochondria radiate. In this confocal image the host cell nuclei are apparent central to an organelle-free cortex, and there are occasional concentrations of mitochondria (orange) distal to these nuclei. Parasite stages are negatively stained by this method and appear dark. The overall appearance suggests that there is some type of MTOC in the centre of the cell that organizes parasite stages, host cell organelles and nuclei down microtubules that radiate out from the centre. In this and similar images of infected cells the calcein distribution was uniform throughout the structure, with a cortex free of organelles and parasites, suggesting that while there was no membrane-bound intracellular partitioning there was a preferential location for the concentration of host cell organelles and parasites. The uniform calcein localisation with the multinucleate structure suggests it was a single cell rather than a cluster of infected cells.

Figs. 4 and 5 show confocal images of immunocytochemically double-labeled multinucleate cells produced after 14 days of infection with *V. corneae*. In these images α tubulin is labeled green and the microsporidia parasite stages are labeled red. In Fig. 4 the α tubulin staining suggests a single, central, large MTOC. Parasite stages (red) can be seen to radiate out from this MTOC. Typically in cells that have been infected for a longer period, the degree of organisation seen in Figs. 1–3 begins to be lost and multiple dense microtubule bundles develop. Fig. 5 shows a whole-cell composite confocal image of an infected cell in which such a dense tubulin bundle has developed. As the multinucleate cells increased in size, smaller MTOCs appeared near the periphery suggesting either a fusion of cells around the xenoma-like structure or resumption of cytokinesis in the areas of the most peripheral host cell nuclei.

To confirm that there was only a single MTOC in the most organized structures, examples of early multinucleate cells were double-labeled for γ tubulin and parasite stages. Images of γ tubulin should identify the location of the MTOC as it is via γ tubulin that the microtubules are attached to the centrioles (Joshi 1994). Fig. 6 shows a fluorescent microscopic image of a *V. corneae*-infected, double-labeled multinucleate cell in which γ tubulin appears green, parasite stages appear red and host cell nuclei and nuclei of adjacent unin-

Table 2. Cell capacitance (pF) of uninfected E6 cells and *Vittaforma corneae*-infected multinucleate E6 cells.

	Mean + SEM	Range
Uninfected cells	21.2 ± 2.1	12.3–32.6
V. corneae-infected multinucleate cells	97.3 ± 12.1	35.8–158.6
Difference	$p < 0.01$	

fected cells were stained with DAPI and appear blue. The punctuate γ tubulin sites locate centrioles adjacent to individual E6 cell nuclei and in a cluster near the centre of the multinucleate cell. Figs. 4 and 6 are consistent with the interpretation that through most of the lifespan of these large multinucleate structures a single MTOC organizes the microtubules, determining the structural orientation of parasite stages and host cell organelles and nuclei.

To determine if these multinucleate cells were single cells as was suggested by the calcein-loaded cells, or were clustered or coalesced infected cells, the whole-cell patch clamp technique (Hamill et al. 1981) was used to measure total membrane capacitance. Membrane capacitance is directly proportional to the plasma membrane surface area. Uninfected E6 cells were readily clamped. However, it was difficult to secure a good seal between the patch pipette and the infected multinucleate cells, suggesting that their surface was either significantly ruffled or covered with debris. Table 2 summarizes the results of some successful seals and subsequent whole-cell mode measurements in which the mean total membrane capacitance of infected cells was increased by an average of five-fold over the mean total membrane capacitance of uninfected cells. Transient values that were just in excess of 200 pF were also obtained. Together with the uniform distribution of calcein, these data suggest that the smaller infected multinucleate structures were single cells. The capacitance values that were obtained, however, were lower than one would predict for the very large size of these multinucleate cells.

Scanning electron microscopic observations of the surface morphology of these multinucleate cells explained the difficulty in obtaining a seal in the patch-clamp experiments. Typically, as the structures increased in size, their surface became covered with spores, polar tubes, debris, and presumably sporoplasm. However, frequently areas of smooth cell surface, the nature of which is still unknown, penetrated the surface debris (Fig. 7).

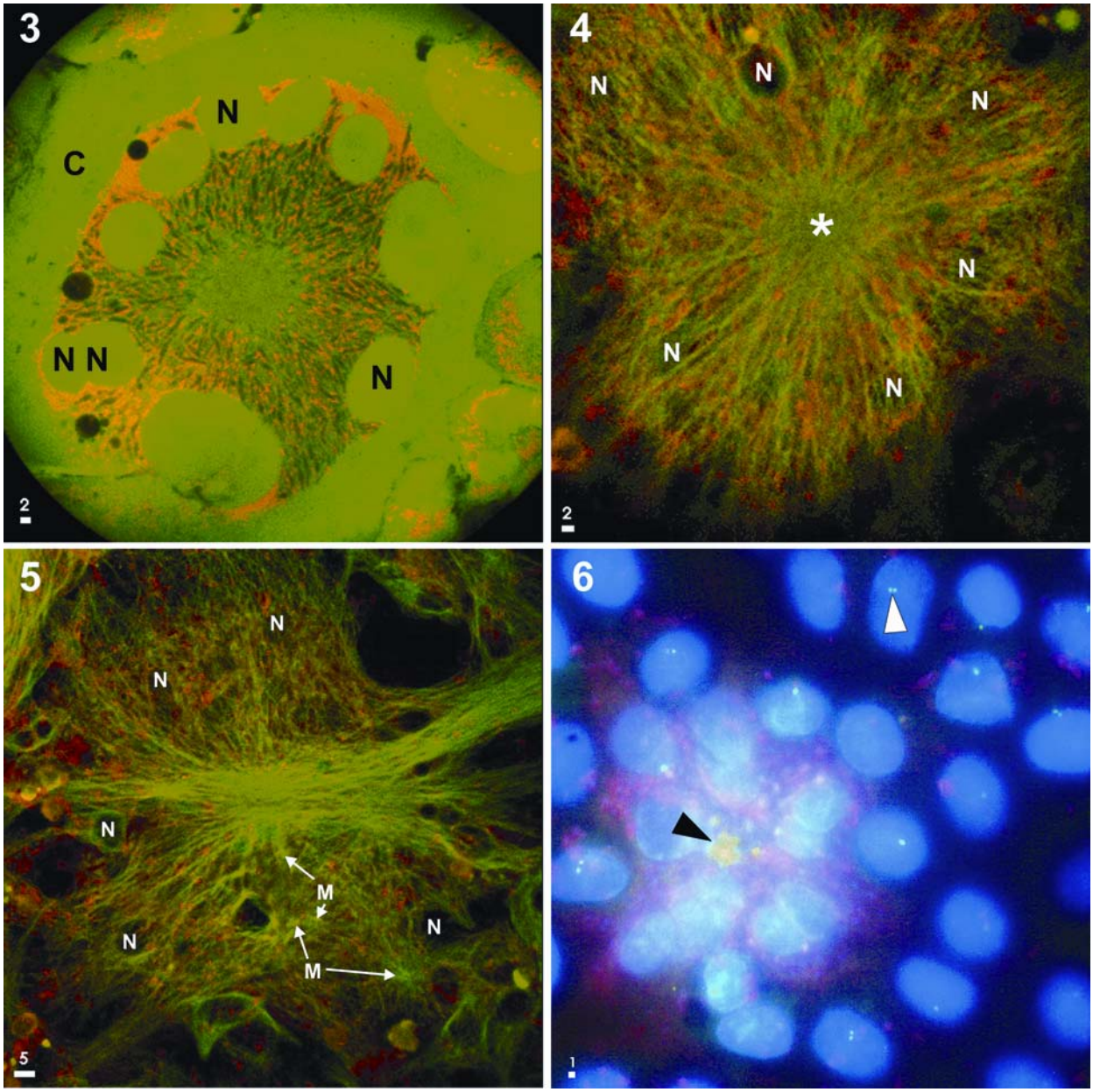
DISCUSSION

Intracellular parasites are well known for their ability to successfully subvert the structure and function of their host cells and thereby optimize their intracellular niche. The array of host-parasite relationships that have evolved has resulted in some very unique host cell structures, such as nurse cells produced by *Trichinella* (Despommier 1993) and xenomas produced by several microsporidia species (Lom and Nilsen 2003). The multinucleate structure described here has the gross appearance of a xenoma. However xenomas are usually mononucleate and often surrounded by fibroblasts and connective tissue.

The development of large cells filled with parasite stages requires significant modification of the host cell cytoskeleton. For example, the *Encephalitozoon* microsporidia develop within a parasitophorous vacuole and to accommodate the parasites, host cell microtubules reorganize around this vacuole (Leitch et al. 1999). In *Toxoplasma gondii*-infected cells the parasitophorous vacuole is stabilized by a sheath of host cell intermediate filaments (Halonen and Weidner 1994). In the present study it appears that the host cell microtubule cytoskeleton played a major role in organizing the *Vittaforma corneae* stages and supporting host cell organelles (Fig. 4). Having mitochondria and developing parasite stages organized by the same cytoskeletal elements helps ensure their proximity to one another and presumably optimizes host cell support of parasite development. This is similar to the situation seen in *Encephalitozoon*-infected cells where mitochondria are organized around the parasitophorous vacuole (e.g. Scanlon et al. 2004) and in *Toxoplasma*-infected cells where both endoplasmic reticulum and mitochondria are in intimate contact with the parasitophorous vacuole (Sinai et al. 1997). In the smaller and most highly organized multinucleate cells there was a clear cortex, devoid of parasites. This observation further suggests that the host cell microtubules dominate parasite distribution, rather than the microfilaments and associated proteins that generally constitute the cortex (Maupin and Pollard 1983).

Vittaforma corneae is a microsporidium that is rarely seen in humans. First named *Nosema corneum* by Davis and colleagues (1990) when found in a corneal stroma infection of an immunocompetent, HIV-negative patient, it was later renamed *Vittaforma corneae* (Silveira and Canning 1995). It was the first human microsporidium to be placed in culture (Shadduck et al. 1990). *V. corneae* causes a disseminated infection when administered intraperitoneally to immunodeficient mice (Silveira et al. 1993), and a *Vittaforma*-like microsporidium has recently been found in immunodeficient and immunocompetent Portuguese patients with diarrhoea (Sulaiman et al. 2003).

In the initial report describing the *in vitro* culturing of *V. corneae* (Shadduck et al. 1990), two epithelial cell lines were used, SIRC (ATCC 60) and MDCK (ATCC 34). While it was noted that host cell mitochondria clustered around the parasites that develop in the host cell cytoplasm, the cells became bloated with parasite stages and then detached. Multinucleate cells of the type



Figs. 3–6. Immunofluorescent images of *Vittaforma corneae*-infected multinucleate E6 cells illustrating the intracellular organisation of parasites and host cell microtubules, mitochondria and nuclei. **Fig. 3.** Confocal image of a calcein-loaded infected multinucleate E6 cell probed with MitoTracker showing the location of the host cell nuclei (N) and the radiation of parasite stages (small dark central objects) and host mitochondria (orange) from a central site, all surrounded by an organelle-free cortex (C). **Fig. 4.** Confocal image of an infected multinucleate E6 cell probed for α tubulin (green) and parasite stages (red) demonstrating radiation of microtubules and parasite stages from a central microtubule organizing centre (MTOC) (*). N – host cell nuclei. **Fig. 5.** Composite confocal image of a larger *V. corneae*-infected multinucleate E6 cell probed for α tubulin (green) and parasite stages (red) demonstrating large bands of microtubules. N – host cell nuclei. M – small MTOCs. **Fig. 6.** Immunofluorescent microscopic image of a *V. corneae*-infected multinucleate E6 cell probed for γ tubulin (green) and parasite stages (red). Nuclei from the infected multinucleate cell and from surrounding mononucleate infected and uninfected cells were stained with DAPI (blue); γ tubulin indicates the location of centrioles (white arrowhead) and the location of the multinucleate cell MTOC (black arrowhead). Scale bars: Figs. 3, 4 = 2 µm; Fig. 5 = 5 µm; Fig. 6 = 1 µm.

reported here were not described. SIRC is of rabbit corneal epithelium origin, but is biochemically more fibroblastic than it is epithelial (Niederkorn et al. 1990), while MDCK cells polarize and differentiate in culture. We have tested several cell lines for their ability to produce highly organized multicellular structures when

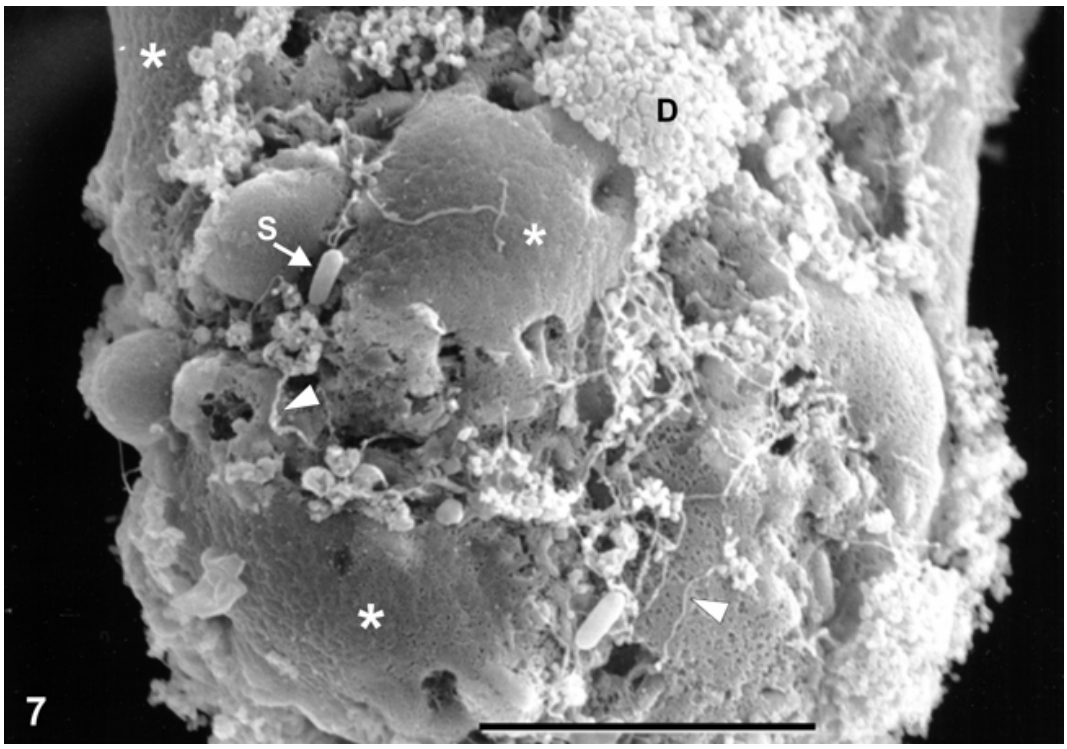


Fig. 7. Scanning electron micrograph of a section of a large *V. corneae*-infected multinucleate E6 cell structure. Parasite spores (S), polar filaments (arrowheads), cell debris and presumed sporoplasm are visible (D), separating areas of cell surface (*). Scale bar = 10 μm.

infected with *V. corneae*. As seen in Table 1, only E6 cells supported such a structure. E6 cells are non-polarized, do not differentiate, and formed stable monolayers that supported infections for over 10 days. Of the cells that did not exhibit multinucleate structures when infected, Caco-2 and HCT-8 spontaneously differentiate and exhibit pronounced polarity, HT-29 is poorly differentiated under the culture conditions used here, and SW-480 is undifferentiated and lacks polarity (Chantret et al. 1988). SW-480 cells are frequently multinucleate. Nevertheless, these cells were unable to support the highly organized multinucleate structures described. SW-480 cells also differ from infected E6 cells in that they detached readily, as did the poorly differentiated HT-29 cells. The Caco-2 cells and the HCT-8 cells differentiate spontaneously and cell loss only occurred from the tips of villus structures. Thus it would appear that for the large multinucleate infected cells to develop, the host cell must be non-polarized, undifferentiated and be able to be maintained in a stable culture with a low rate of apoptosis/necrosis.

The early, well-organized multinucleate structures produced when *V. corneae* infects E6 cells (Figs. 1–3) suggested that nuclear division continued in the absence of cytokinesis. As the parasite load increased and the multinucleate cells continued to enlarge, cells appeared to have fused or cells and cell debris coalesced with the structure's surface (Figs. 5, 7). The early multinucleate structures were clearly single cells as determined by calcein loading experiments (Fig. 4) and membrane capacitance measurements (Table 2). While the infected cell membrane capacitance values were large enough to account for the early multinucleate structures being single cells, the values were not large enough to support the concept that the very large structures were single cells. However the difficulties encountered patching these larger structures may have skewed the results.

We were unable to determine if the multinucleate infected E6 cells were the result of continued nuclear division without cytokinesis, fusion of adjacent infected cells, or a combination of both. If the multinucleate cells were the result of continued mitosis in the absence of cytokinesis, one would expect to observe occasional mitotic spindles, which we did not. While there is only limited information available on the effects of microsporidial infection on the cell cycle, that which is available favours the inhibition of host cell mitosis (e.g. Scanlon et al. 2000). On the other hand, if cell fusion was the cause of the multinucleate cells, one would expect to have seen examples of partial fusion, which we did not observe. Multinucleate giant cells have long been known to result from monocyte cell fusion. This is an inflammatory response seen in many granulomas. In the Langerhans type of multinucleate giant cell the centrosomes of the fused cells locate centrally, giving rise to a central MTOC (Cain et al. 1981), similar to the

cells reported here (Fig. 6). It is possible that the multinucleate infected cells we describe were a combination of this type of cell fusion, together with some mitosis without cytokinesis. The latter could be promoted by the loss of centrioles to a central MTOC. For example, Salisbury and colleagues (2002) used a centrin-2 "knockdown" method to show that with depletion of one or both centrioles the nuclei continued to divide for a limited time but cytokinesis was inhibited, resulting in multinucleate cells. After several nuclear divisions the cells died. If the multinucleate infected cells are indeed similar to inflammatory multinucleate giant cells and are the result of cell fusion, this would explain why the multinucleate SW-480 cells did not result in the high level of organisation of parasites and host cell nuclei described here. In cancer cell lines, such as SW-480, the multinucleate morphology is the result of continued mitosis and not fusion.

Whatever the cause of the multinucleate nature of these infected cells, it is clear that microtubules dominated the organisation of parasites and host cell organelles (Figs. 4–6). Centrioles from the multiple host cell nuclei coalesced (Fig. 6) to organize a single centrally located MTOC from which radiated microtubules (Fig. 4). Host cell mitochondria and parasite stages oriented themselves along the microtubules, and together with the host cell nuclei they were gradually moved outwards towards the plus end. This is one explanation for the more peripheral concentration of host cell nuclei, groups of mitochondria and occasionally clusters of parasite stages (Figs. 4 and 5). Another explanation is that the nuclei from fused infected cells, together with some of the cell's mitochondria and parasite stages remained at the periphery of the cell mass.

The present study describes the formation of large multinucleate cells when E6 cells are infected with *V. corneae* and in which single central MTOCs organize microtubules to orient parasites and supporting host cell organelles. Such a structure provides an environment that protects parasite development against the host immune response, much as is the case in a xenoma. Because of the limited number of clinical reports of *V. corneae* infections it has yet to be determined if this type of multinucleate structure is found *in vivo* or is unique to infected undifferentiated cultured epithelial cells.

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