Behaviour and pathogenicity of Trichomonas vaginalis in epithelial cell cultures

A study by light and scanning electron microscopy

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SUMMARY   The behaviour and pathogenic effects of *Trichomonas vaginalis* in mammalian cell cultures were studied using light microscopy and scanning electron microscopy. Six hours after inoculation of the parasites into the cell cultures about 10% of the epithelial monolayer was destroyed. The parasites adhered to the epithelial cells, developed an amoeboid morphology, and crawled over and under the monolayer of cells. These observations suggest that the adhesiveness, amoeboid morphology, and motility of *T vaginalis* may be important mechanisms in the injury caused to the vaginal epithelium.

Introduction

It is widely recognised that many strains of the human urogenital protozoan *Trichomonas vaginalis* are inherently pathogenic and are capable of causing serious disease, especially in women. The fact that axenically cultured *T vaginalis* can elicit extensive cytopathogenic and histopathogenic changes on inoculation into laboratory rodents[1-3] and into cell and tissue cultures[4-8] gives clear evidence that the parasite is a potential primary pathogen of the human urogenital tract.

However, it is also clear that at present little is known of the mechanisms involved in the pathogenicity of *T vaginalis*. Studies at the light microscope[9] and electron mocroscope[10-13] levels of the interaction of the trichomonad with human vaginal and cervical epithelial cells have added considerably to our understanding of the behaviour of *T vaginalis* in its natural host. Nevertheless, such approaches offer little opportunity for direct observation of the living parasites and have the disadvantage of the possibility that the parasite behaviour may be modulated by host conditions such as the menstrual cycle or pregnancy.[14 15] In contrast, the use of cell or tissue cultures offers a means for direct and controlled fine-structural and behavioural analysis of the inherent mechanisms of the pathogenicity of *T vaginalis*.

This paper describes the interaction of *T vaginalis* with cultured mammalian epithelial cells as seen by light and scanning electron microscopy; a parallel transmission electron microscope study was undertaken and the results will be presented separately.

Materials and methods

T VAGINALIS

The trichomonads used in this study were derived from a cryopreserved stock of *T vaginalis* (code No LUMP 889) held in the department of medical protozoology at the London School of Hygiene and Tropical Medicine. The history of this stock is given elsewhere,[16] and the methods used for the isolation from vaginal secretion, laboratory culture, and cryopreservation have also been described.[16-19]

T vaginalis were removed from cryopreservation as required and cultured in a 15-ml bottle of *Trichomonas* medium[18 20] to a parasite density of about 10^5/ml. The trichomonads were harvested by centrifugation at $250 \times g$ for 10 minutes and resuspended in fresh culture medium.

EPITHELIAL CELLS

Rabbit kidney tubule epithelial (RK 13) cells (purchased from Gibco Ltd, Glasgow) were cultured on one side of flat-sided 50-ml glass bottles in Medium 199 supplemented with 10% fetal calf serum, 25 mmol/l HEPES buffer, and 100 units per

ml penicillin and 50 units per ml streptomycin (all culture media obtained from Wellcome Ltd, Beckenham). Cells were passaged weekly. For experimental use, confluent monolayers of epithelial cells were grown on glass coverslips (22 mm in diameter) in plastic screw-capped jars (Sterilin Ltd, Richmond).

INOCULATION OF CELL MONOLAYERS
The culture medium was decanted from the RK13 cell monolayers and replaced with 5 ml of a medium consisting of 2 parts *Trichomonas* medium and 1 part Medium 199 (plus supplements) containing 4×10^5 *T vaginalis* per ml. The pH of this medium mixture was 6·8, and preliminary experiments showed that it supported normal growth of both *T vaginalis* and RK 13 cells during the periods of experimentation. The infected cultures were incubated at 37°C for up to 48 hours. The results collated here were obtained from more than 50 infected cultures.

LIGHT MICROSCOPY
Living cultures
For the first six hours after inoculation of the epithelial cells with *T vaginalis* the cultures were removed from the incubator at 30-minute intervals for 2-3 minutes to examine the state of the cultures with a phase-contrast inverted microscope. Subsequently, observations on living cultures were made at longer intervals for another 42 hours.

Fixed cultures
Infected cultures were fixed at intervals between 30 minutes and 36 hours after infection. The medium was carefully decanted and the cultures fixed in absolute methanol at room temperature for 10 minutes, stained with Giemsa, dried in air, and mounted on slides in Euparal Vert.

SCANNING ELECTRON MICROSCOPY
Infected cultures were fixed for scanning electron microscopy six hours after infection. The medium was carefully decanted and replaced with 3% glutaraldehyde in 0·066 mol/1 sodium cacodylate buffer, pH 7·2, at room temperature for one hour. The cultures were then rinsed in buffer for 30 minutes, post-fixed in 1% osmium tetroxide in buffer at room temperature for one hour, then dehydrated in acetone, and critical-point dried from liquid CO_2. The coverslips were broken into small pieces of about 10 mm², coated with a 40-nm layer of gold, and examined with a Cambridge Instruments Stereoscan S4.10 electron microscope operating at 20 kV with a tilt angle of 45°.

Results

LIGHT MICROSCOPY
Epithelial cell cultures
Although the RK 13 epithelial cells used in this study were of rabbit, and not human, origin they were nevertheless very similar in morphology and behaviour in culture to human ectocervical cells.[21] The RK 13 cells grew to form a non-dividing confluent monolayer of polygonal cells (fig 1). Some of the monolayers contained a few binucleated and multinucleated cells; in addition, some cells contained large empty vacuoles. These morphological abnormalities were present in controls and *T vaginalis*-infected cultures and so were not included when assessing the injurious effects of the parasites.

### BEHAVIOUR OF *T VAGINALIS* IN CELL CULTURES
Each monolayer of RK 13 cells was inoculated with 2×10^5 *T vaginalis*. This inoculum was similar to those used in earlier studies on the parasite in cell cultures[7 8]; furthermore, with this inoculum large areas of cell lysis, suitable for microscopical analysis of parasite behaviour, occurred during the first 10 hours of infection and before culture conditions, such as pH and nutrient level, had changed to the detriment of cells or parasites.

At 30 minutes after inoculation most of the trichomonads had settled on to the epithelial cell monolayer and were actively swimming over the cells. By one hour after inoculation many of these had aggregated into large clumps of up to 200 individuals (fig 2). This parasite behaviour has been reported previously and is called "rosetting"[4] and "swarming".[6]

After one to two hours the aggregates of *T vaginalis* and surrounding individual organisms (fig 2) lay motionless on top of the epithelial cells; only their flagella and undulating membranes showed movement. Gentle rocking of the culture vessels did not detach the trichomonads, showing that they were adhering to the cell monolayer.

The first signs of the injurious effects of *T vaginalis* on the epithelial monolayer were observed between two and three hours after infection. Small cell-free areas ("lesions"[6 8]) appeared beneath or to one side of the largest clumps of parasites (fig 2). No lesions were seen beneath the individual parasites that were adhering to the monolayer nor were lesions present in the unparasitised areas of the monolayers. Gradually more and more lesions developed beneath the clumped parasites, which then settled into the cell-free areas.

After six to 10 hours about 10% of the area of the monolayers was destroyed and the cultures were
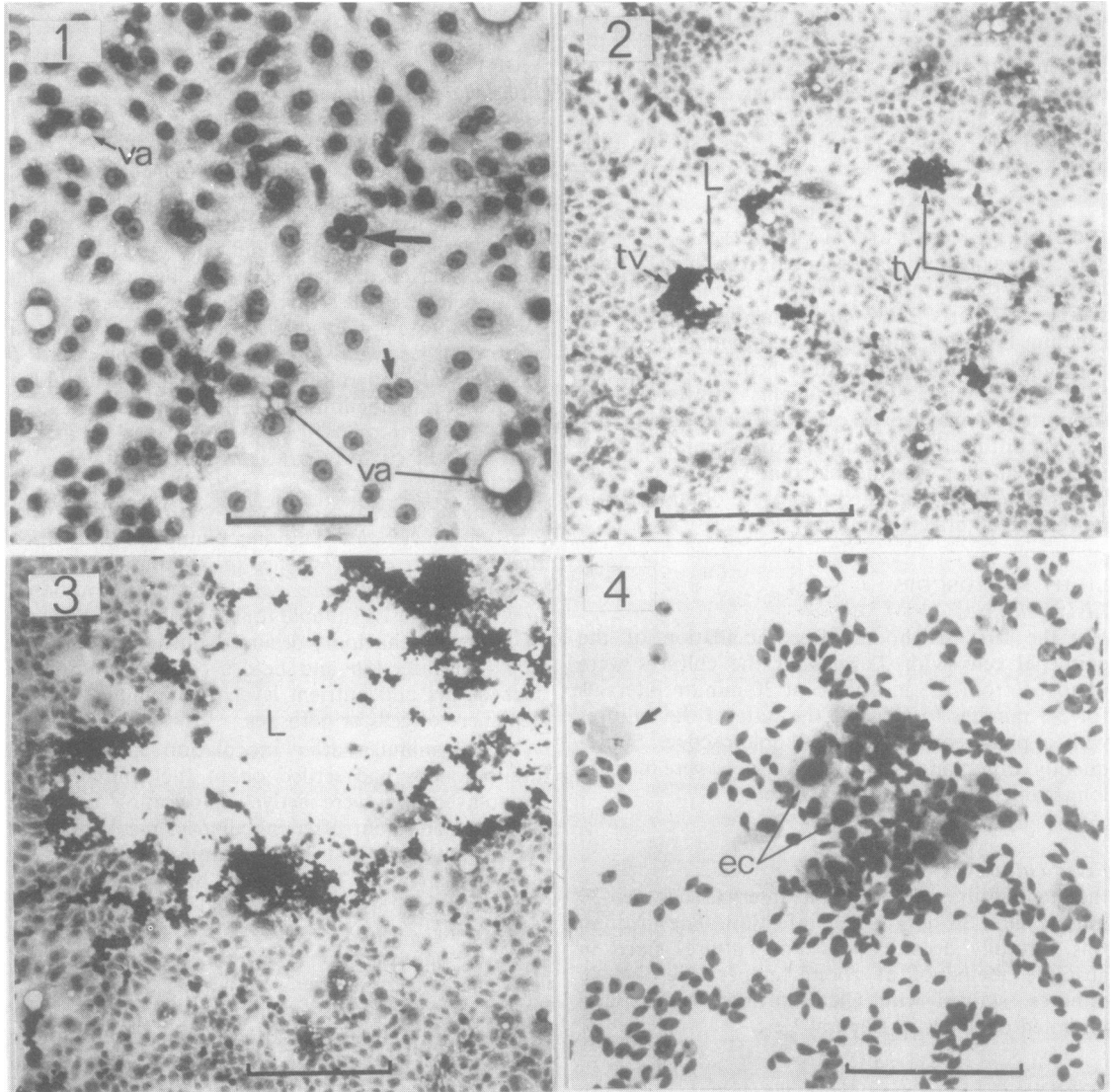
FIGS 1-4 *Light micrographs of confluent monolayer of RK epithelial cells. Giemsa-stained. (1) Some of the cells contain more than one nucleus (arrows) or vacuoles (va) (scale bar = 100 μm). (2) At two hours after inoculation with T vaginalis, aggregated and individual darkly stained parasites (tv) adhere to monolayer (small lesion (L) lies to the right of the largest aggregate) (scale bar = 250 μm). (3) At 10 hours after inoculation, many parasites are palisaded against the cells lining a large lesion (L); the unparasitised areas of the monolayer are free of lesions and appear normal (scale bar = 100 μm). (4) At 36 hours after inoculation, numerous parasites adhere to the exposed coverslip and to a few remaining cells (ec); note the abnormally large trichomonad (arrowed) (scale bar = 100 μm).*

dotted with lesions up to 1 mm wide (fig 3). Generally the centres of the lesions contained few parasites; the trichomonads moved outwards as the lesions enlarged, remaining in close contact with the epithelial cells lining the lesions' edge. Almost all of the trichomonads adhering to the epithelial cells or to the glass substratum were highly amoeboid, and in some instances amoeboid *T vaginalis* were seen to crawl underneath the monolayer for distances of up to 100 μm from the edge of a lesion.

After six hours the pH of the medium of the infected cultures was not lower than 6·4; thus it was

improbable that increased acidity of the culture medium was a contributing factor in the formation of the lesions.[8] [22]

From 10 hours onwards the lesions enlarged and coalesced until at about 36 hours after inoculation most of the epithelial cell monolayer was destroyed (fig 4). At 36 hours the medium contained much debris from the lysed cells, and the cultures contained large numbers of trichomonads swimming in the medium and crawling on the coverslips. Typically, the parasite density in the cultures, including adherent organisms, was between 1 and 2×10^6 trichomonads per ml, and the pH of the medium was between 4·5 and 5·0; thus medium acidity was undoubtedly an important factor in cell lysis in the later stages of the infection.[22]

SCANNING ELECTRON MICROSCOPY
T vaginalis
The presence of large numbers of adherent *T vaginalis* in the lesions presented an opportunity for a scanning electron microscope (SEM) study of not only the surface structures of the parasite at high resolution but also the morphological adaptations which followed adhesion of *T vaginalis* to glass and to the surface of an epithelium.

An example of an amoeboid *T vaginalis* is shown in fig 5; the rounded body, bearing the flagella and undulating membrane, lies above a single broad flat pseudopodial process. The upper surfaces of the pseudopodia were generally fairly smooth with few processes, but their edges frequently had short filopodial extensions, about 0·5 μm wide and between 2 and 5 μm long. Commonly the tips of the filopodia adhered to the substratum (fig 5). A second, more flattened, amoeboid trichomonad is shown in fig 6.

The major surface structures of *T vaginalis* are shown in figs 6-8. The four anterior flagella, the fifth recurrent flagellum, and the undulating membrane emerge from the anterior pole (figs 6 and 7). In some organisms the bases of these organelles lay in a shallow depression (fig 7), which is similar to the flagellar pocket of kinetoplastid flagellates.[23] But since this structure was not present in all the trichomonads examined (for example, see fig 15) it may be that the pocket is not a permanent structure and occurs only as a result of pseudopodial protrusion around the bases of the flagella. The undulating membrane is a thin fold of cytoplasm which projects from the body for 1 μm; the recurrent flagellum is attached to the membrane and lies parallel to, and
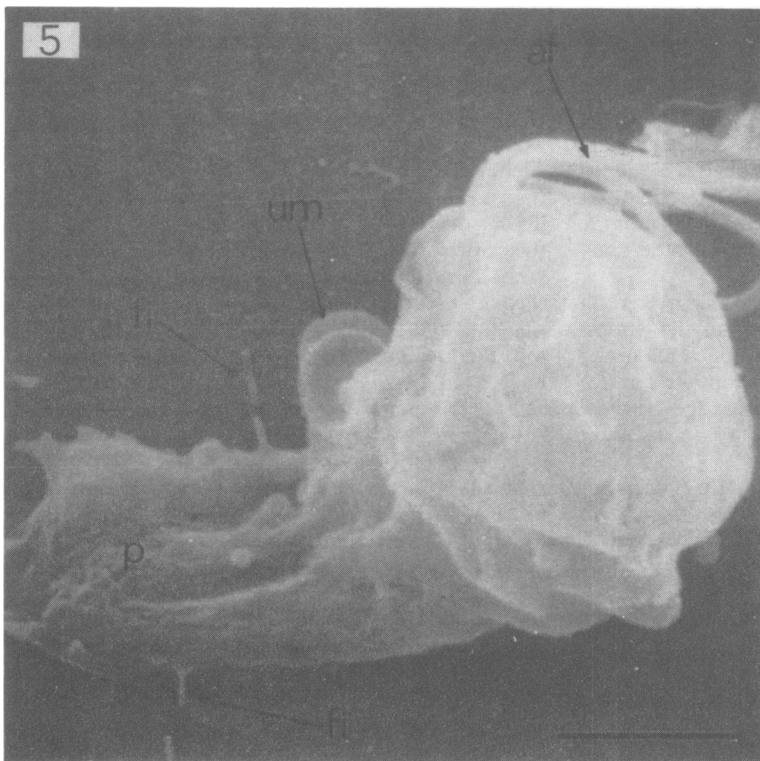

FIG 5 *Scanning electron micrograph of an amoeboid T vaginalis adhering to a glass coverslip in the centre of a lesion. Note the broad pseudopodium (p) from which small filopodia (fi) (which have fractured during preparation) extend down on to the glass surface. The anterior flagella (af) and part of the undulating membrane (um) emerge from the main body mass which projects up into the medium. Scale bar = 2 μm*
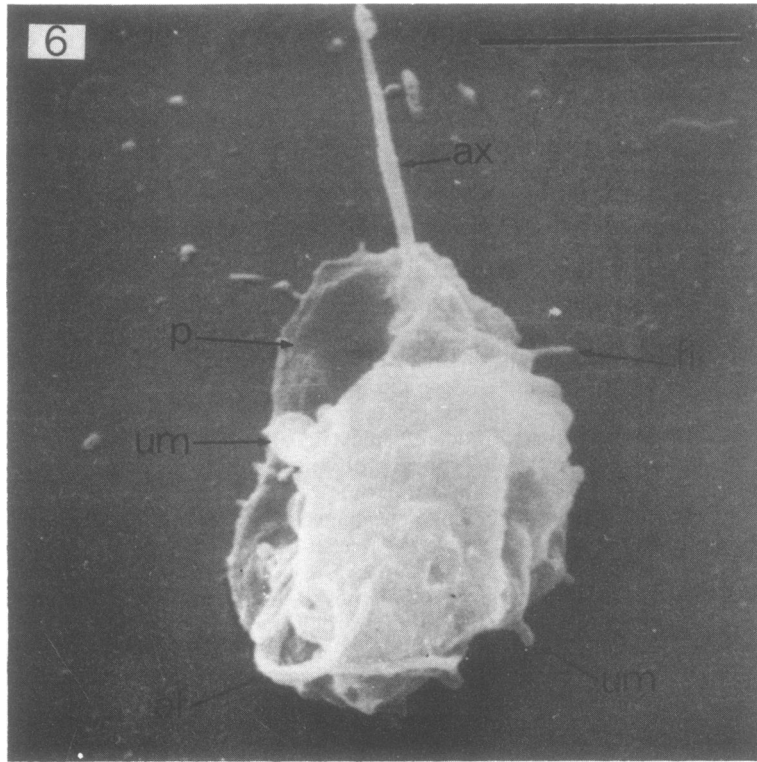
FIG 6   *An amoeboid T vaginalis adhering to a glass substratum. This trichomonad is in an early stage of binary fission; two undulating membranes (um) are seen on each side of the body. The axostyle (ax) projects from the posterior pole. Note the large smooth-surfaced pseudopodium (p) along one side of the body. Scale bar = 5 μm*

about 0·5 μm from, its free edge (fig 8). Together these two structures pass posteriorly and terminate about halfway down the trichomonad (figs 6 and 9). The axostyle protrudes from the posterior pole (fig 6); it is a tapering structure with a membrane continuous with the plasma membrane of the body and reaches a length of up to 10 μm.

Many of the trichomonads in the lesions were in various stages of binary fission. One of the earliest signs of division in *T vaginalis* is the replication of the anterior flagella and undulating membrane (fig 6). Subsequently, a longitudinal cleavage furrow appears and eventually separates the two daughter trichomonads (fig 9).

Very occasionally abnormally large (fig 4) or multi-flagellated (fig 10) forms of *T vaginalis* were observed. Similar forms have been seen before in this laboratory and elsewhere,[24][25] and although their significance is not clear they may simply be degenerating forms, deficient in the mechanisms of cytokinesis.

INTERACTION OF *T VAGINALIS* WITH CELL CULTURES
In the RK 13 epithelial cell cultures the normal morphology of those cells in and around the lesions was considerably changed after their attack by the trichomonads (fig 11). Within each lesion most parasites were palisaded against the cells lining the lesions or were adhering to the few remaining cells in the centre of the lesion (figs 12 and 13). The parasites were markedly amoeboid and adhered to the cells through pseudopodial processes, leaving their bodies, flagella, and undulating membranes projecting up into the medium. Many of the parasites appeared to have been crawling over the surfaces of epithelial cells at the time of fixation of the cultures for SEM. A trichomonad attached to the upper surface of a cell is shown in fig 14. In this case pseudopodial activity is most prominent at the anterior pole. However, most of the surface of *T vaginalis* was capable of developing pseudopodia; in fig 15 large pseudopodia are present at the posterior pole each side of the axostyle.

Where parts of the monolayer were lifted up away from the substratum, it was possible to see amoeboid parasites lying between the monolayer and the glass substratum (figs 13 and 16). The ability of the amoeboid trichomonads to migrate underneath the cells suggested that the protrusive activity associated
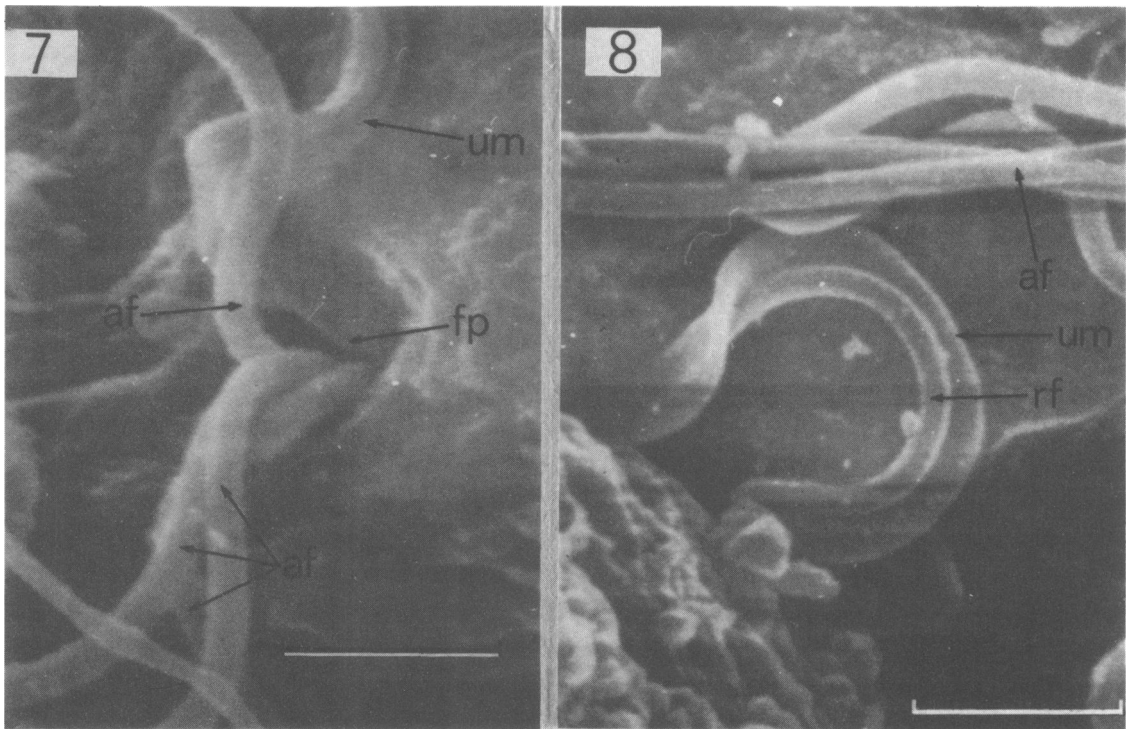
FIG 7 *The anterior pole of T vaginalis showing the points of emergence of the four anterior flagella (af), the fifth recurrent flagellum, and the undulating membrane (um) from the flagellar pocket (fp). Scale bar = 1 μm.*
FIG 8 *The mid-body of T vaginalis showing the anterior flagella (af) and the recurrent flagellum (rf), which is attached about 0·5 μm from the free edge of the undulating membrane (um). Scale bar = 1 μm*

with crawling was partly responsible for the detachment of the epithelial cells from the culture substratum (figs 16 and 17).

PATHOLOGICAL CHANGES IN CELL CULTURES
An important finding in the cell cultures was that morphologically normal epithelial cells, free of attached amoeboid parasites, often lay adjacent to other cells which were damaged and which bore parasites (fig 3). This demonstrated the very local nature of the pathological effects of *T vaginalis* and also indicated that contact between parasite and epithelial cell was an important factor in cytopathogenesis.

One of the most frequent surface changes in the epithelial cells was a loss of the normal flattened shape; the peripheral cytoplasm was frequently retracted, causing a more rounded cell form, from which extended retraction fibres (fig 18). A further change was the loss of microvilli (fig 19). This change may have been caused by an increased permeability of the membrane after injury to osmotic control mechanisms; an increase in cell volume would require additional plasma membrane, which could be recruited from that of the microvillar surfaces.

These pathogenic changes led ultimately to rupture of the plasma membrane of the epithelial cells and the release of cytoplasmic constituents into the medium (fig 19).

Of course, with SEM it was not possible to examine internal fine structural changes in the cell cultures. Accordingly, a parallel transmission electron microscope study was undertaken.[16] Briefly, this showed that adhesion of *T vaginalis* to the epithelial cell surface was followed by fine structural changes, which included mitochondrial and nuclear swelling, a decrease in cytoplasmic density, and a marked vesiculation of the cytoplasm.

Discussion

One of the most interesting aspects of the behaviour of *T vaginalis* in the RK 13 epithelial cell cultures was the ability of the parasite to adhere to the cells and culture substratum and then develop an amoeboid morphology and crawl. These aspects of *T vaginalis* have been noted earlier in light microscope studies of
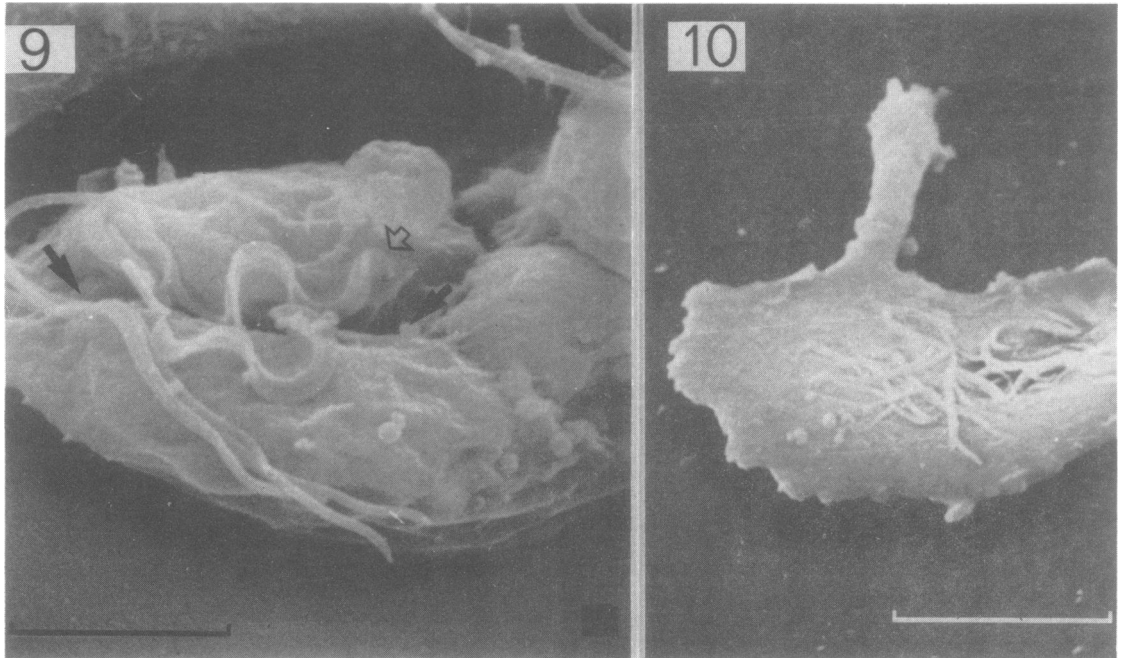
FIG 9 *Binary fission in T vaginalis. A longitudinal cleavage furrow (between closed arrows) separates two daughter trichomonads. Both daughters have undulating membranes which terminate, with the recurrent flagellum, about halfway along the body (open arrow). Scale bar = 4 μm*

FIG 10 *An abnormal disc-shaped T vaginalis with a single long pseudopodium. Five sets of flagella are present on the upper surface. Scale bar = 10 μm*

the pathogenicity of *T vaginalis* in a variety of avian and mammalian cell cultures,[4-8] [16] but this is the first SEM study of the amoeboid parasites and their interaction with epithelial cells.

Many strains of *T vaginalis* are known to form stable adhesions with a variety of substrata including cultured cells and other *T vaginalis*,[4] [6] glass,[20] [26] [27] and tissue-culture-grade plastic.[27] As this study shows, adhesion leads to the development of pseudopodial and filopodial processes giving the organisms a form quite distinct from that which it assumes when suspended—that is, spherical or ovoid.[15] [28] Furthermore, there is good reason to suspect that the adhesiveness and amoeboid form of *T vaginalis* may make significant contributions to the mechanisms of pathogenicity of the parasite in natural human infections. In this study it was found that damage to the epithelial cell monolayer occurred mainly in those areas of contact between amoeboid parasites and the cells. Recent SEM[13] and TEM[10-13] studies on material collected from women with trichomonal vaginitis have provided clear evidence that amoeboid forms of *T vaginalis* are also found in situ: pseudopod-bearing forms, similar to those described in this study, were found clinging to the

superficial layers of the vaginal epithelium and to desquamated cells in vaginal discharge. Moreover, Honigberg and his colleagues[8] [15] have reported that there appears to be a direct correlation between the level of pathogenicity of two strains of *T vaginalis* in women and in cell cultures with the adhesiveness and amoeboid morphology of the strains.

How, then, can these behavioural adaptations of *T vaginalis* contribute to the pathogenicity of the parasite? At present we have few details of the precise mechanisms whereby *T vaginalis* injures cells and tissues. The existence of parasite-derived cytotoxic factors is suggested by reports that parasite-free filtrates of *T vaginalis* cultures can elicit pathogenic changes in cell cultures similar in nature to those produced in the presence of the trichomonads.[4] [8] But other workers have been unable to demonstrate any cytotoxicity of filtrates.[5-7] The reason(s) for this apparent discrepancy are not clear; lability of the toxic factors may be important.[8] Clearly there is a need for analysis and characterisation of the putative toxic factors in *T vaginalis* culture filtrates and of how their activity varies between strains of the parasite differing in their pathogenicity. These factors may include acidic
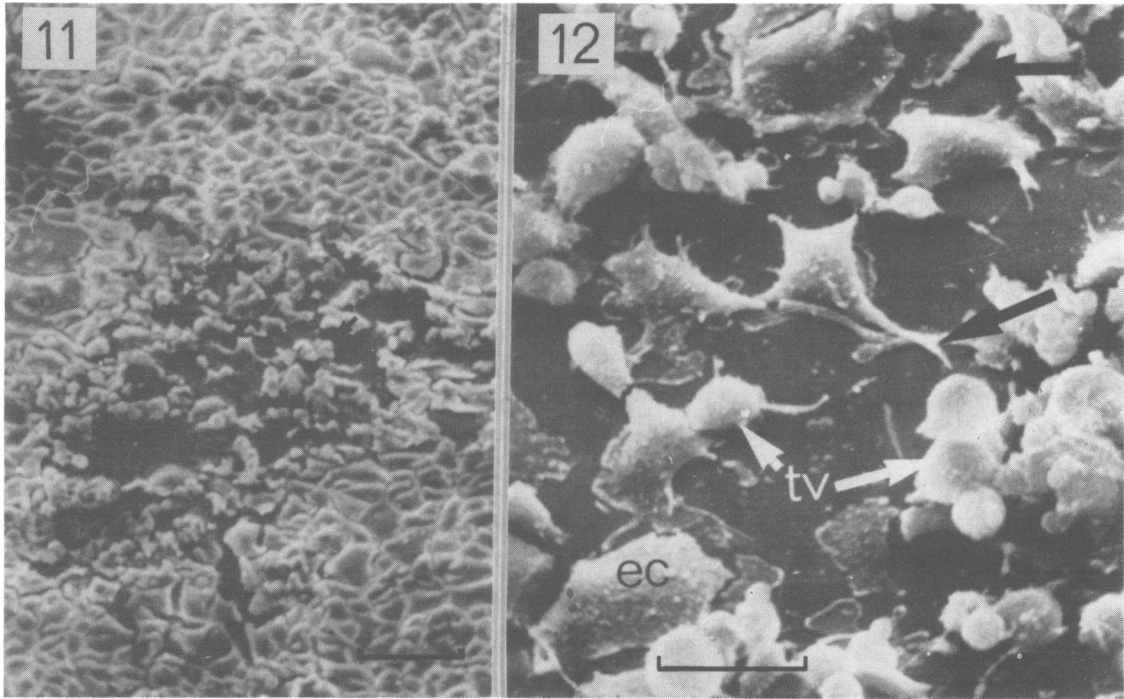
FIG 11   *Scanning electron micrograph of a lesion in an epithelial cell monolayer six hours after inoculation with T vaginalis. Scale bar = 100 μm*

FIG 12   *Higher magnification detail of the lesion shown in fig 11. The lesion contains isolated epithelial cells (ec) some of which possess retraction fibres (arrows). Trichomonads (tv) are adherent to some of the cells. Scale bar = 25 μm*

products of the parasite's metabolism, such as lactic acid,[15] or secreted hydrolytic digestive enzymes, such as hyaluronidase,[29] glycosidases, and acid phosphatase.[16 30] Thus adhesiveness may be a mechanism for increasing the efficiency of parasite-derived cytotoxic and cytolytic factors since they could be applied at short range at the interface between a cell and an adhering trichomonad.

The amoeboid movements of *T vaginalis,* acting alone or in concert with secreted factors, also provide a mechanism for injuring cells and tissues. This study shows that the crawling activity of the parasites may be important in disrupting a cell monolayer; similarly, in a cinemicrographic study of *T vaginalis* in HeLa cell cultures, Christian *et al*[6] observed amoeboid parasites moving over and under the cells, dislodging them from the glass substratum; they concluded that the strenuous (sic) trichomonal activity could be a major factor in cell destruction. The possibility of a purely mechanical mechanism of cell injury was also considered by Nielsen and Nielsen[12]; in a fine structural study of vaginal biopsy specimens they found amoeboid parasites attached to the epithelium, and they postulated that mechanical damage to the epithelial cell membranes could result

from tensions generated by the parasites during movement.

In conclusion, although there clearly is scope for further study to elucidate the mechanisms of pathogenicity of *T vaginalis,* studies such as the present one on the behaviour of the parasite in mammalian cell and tissue cultures have indicated that there may be both mechanical and chemical components operating in natural human infections. The adhesion of trichomonads to the vaginal and ectocervical epithelia, and their protrusive activity during amoeboid crawling, may be important in disrupting the superficial layers of the squamous epithelium and in causing, to some degree, the necrosis and increased rate of desquamation of the vaginal epithelium, which are common features of severe trichomonal vaginitis.[9] But, as others have already pointed out, infected women also frequently show a marked increase in sub-epithelial vascularity of the vaginal and cervical walls[9] which is often out of proportion to the numbers of trichomonads that are found adhering to the epithelium[9 12]; so it is likely that diffusable chemical factors, produced directly or indirectly by the trichomonads, are also involved in the pathology of trichomoniasis.
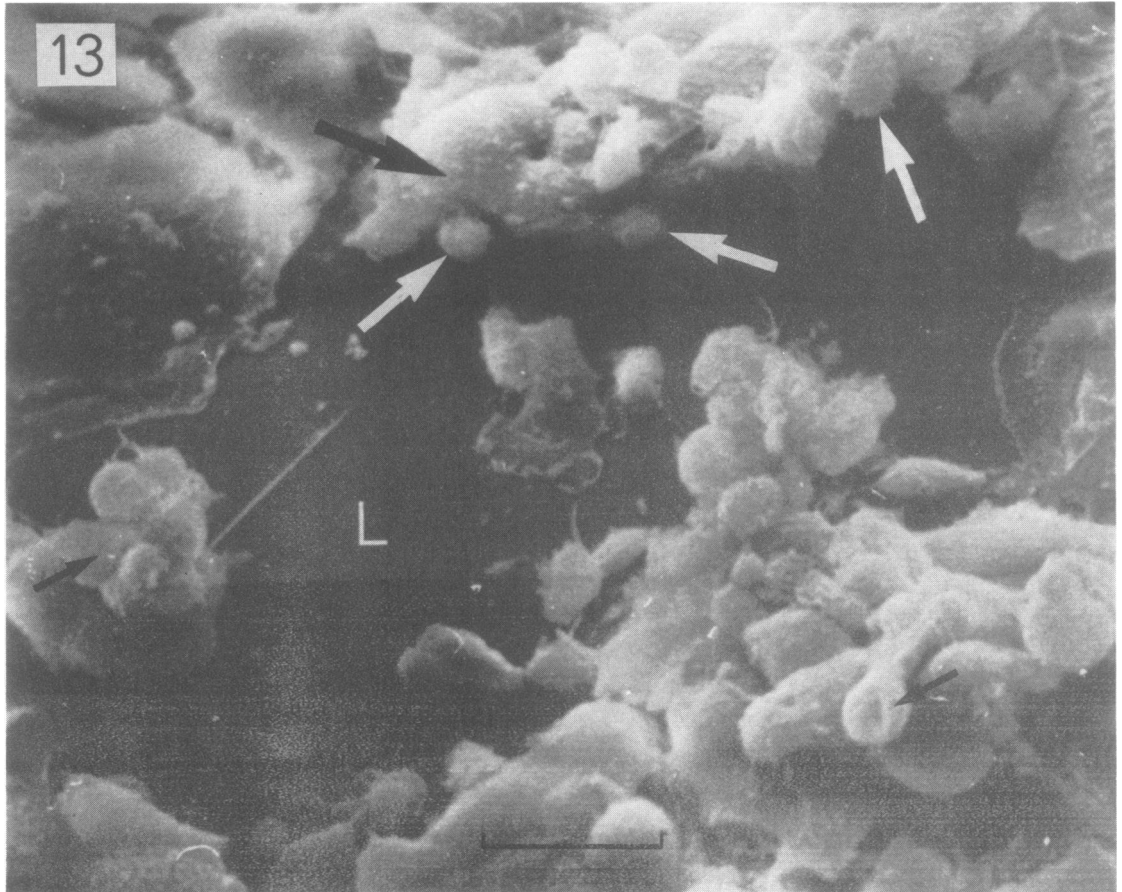
FIG 13   *A lesion (L) in an epithelial cell monolayer six hours after infection with T vaginalis. Groups of parasites (small black arrows) are adherent to the cells lining the lesion. Note the parasites (white arrows) attached to the sides and lower surface of part of the monolayer which is lifted off the substratum (large black arrow). Scale bar = 20 μm*

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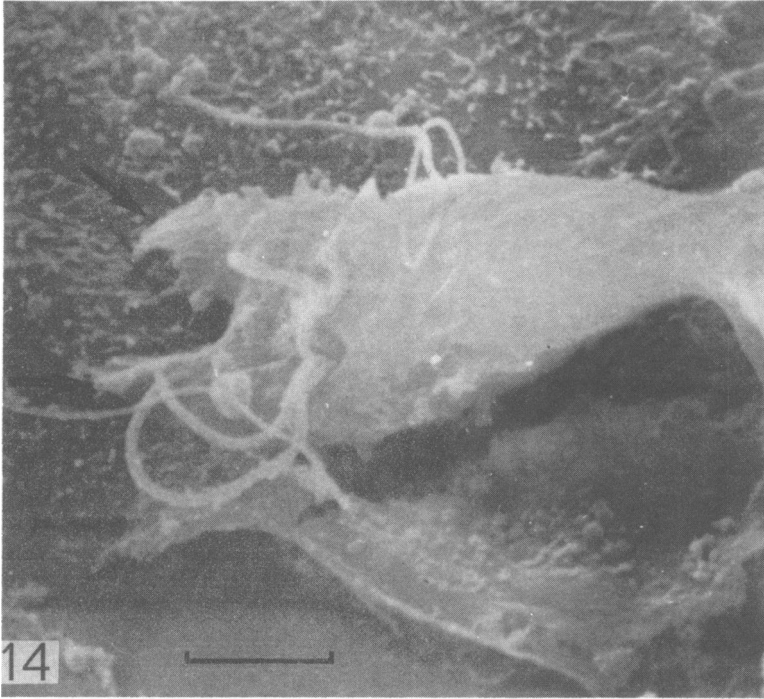
FIG 14 *T vaginalis adhering to the upper surface of an epithelial cell. Note the two short pseudopodia (arrows) at the anterior pole. Scale bar = 2·5 μm*
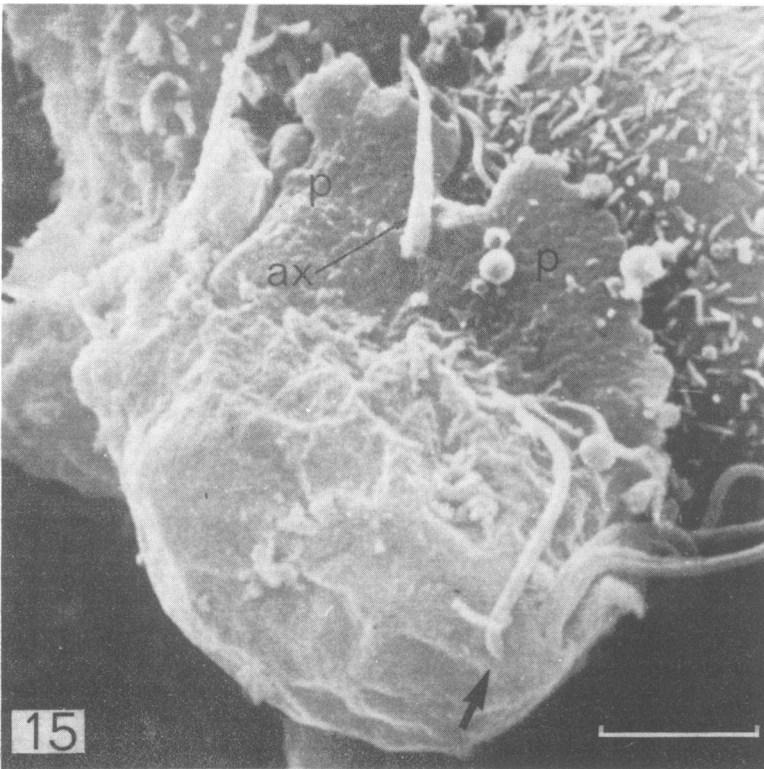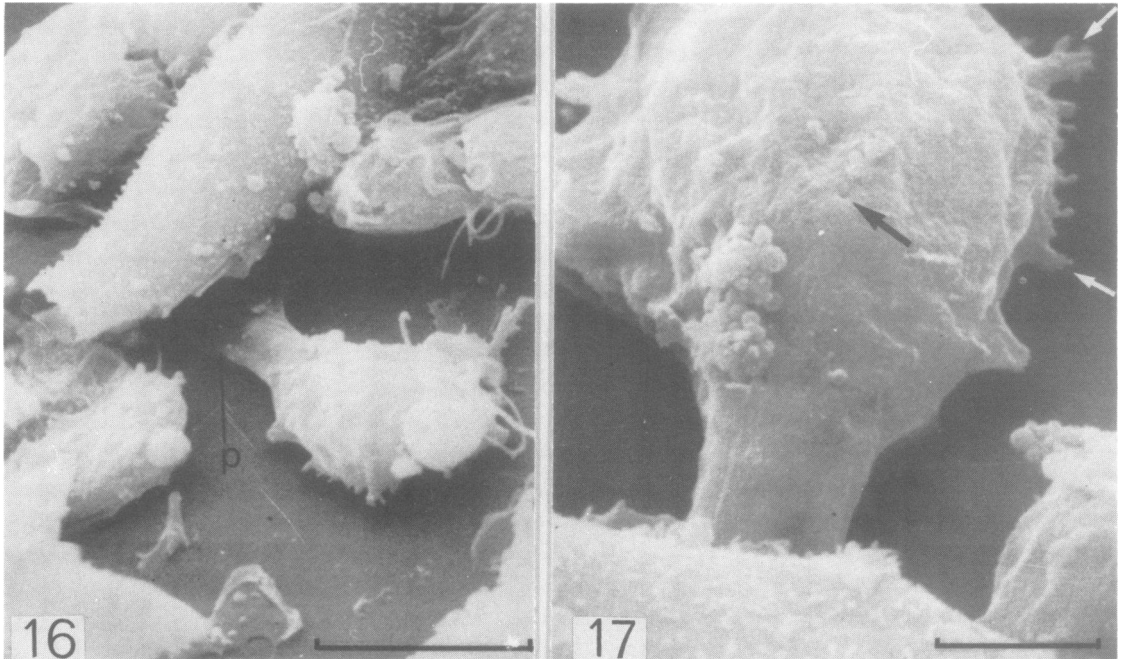

FIG 15 *An amoeboid T vaginalis crawling over the surface of a rounded-up epithelial cell. Two large pseudopodia (p) are in close contact with the cell surface. Note that this trichomonad does not have a flagellar pocket and that one of the anterior flagella (arrow) emerges from the body a short distance from the others (ax = axostyle). Scale bar = 2·5 μm*

FIGS 16 and 17   *Protrusion of T vaginalis pseudopodia underneath epithelial cells. In fig 16 a large pseudopodium (p) lies beneath a cell (the left side of the cell has fractured during preparation). Fig 17 shows the same trichomonad at higher magnification but the specimen has been rotated in the SEM through approximately 150°. The large pseudopodium is protruding beneath the epithelial cell in the foreground. At the rear of the trichomonad are smaller filopodial processes (white arrows). Note the smooth surface of the pseudpodium and body of the parasite; a pinocytotic pit is arrowed. Small vesicles from lysed cells are scattered over the parasite's surface. Scale bars: fig 16 = 10 μm; fig 17 = 3 μm*

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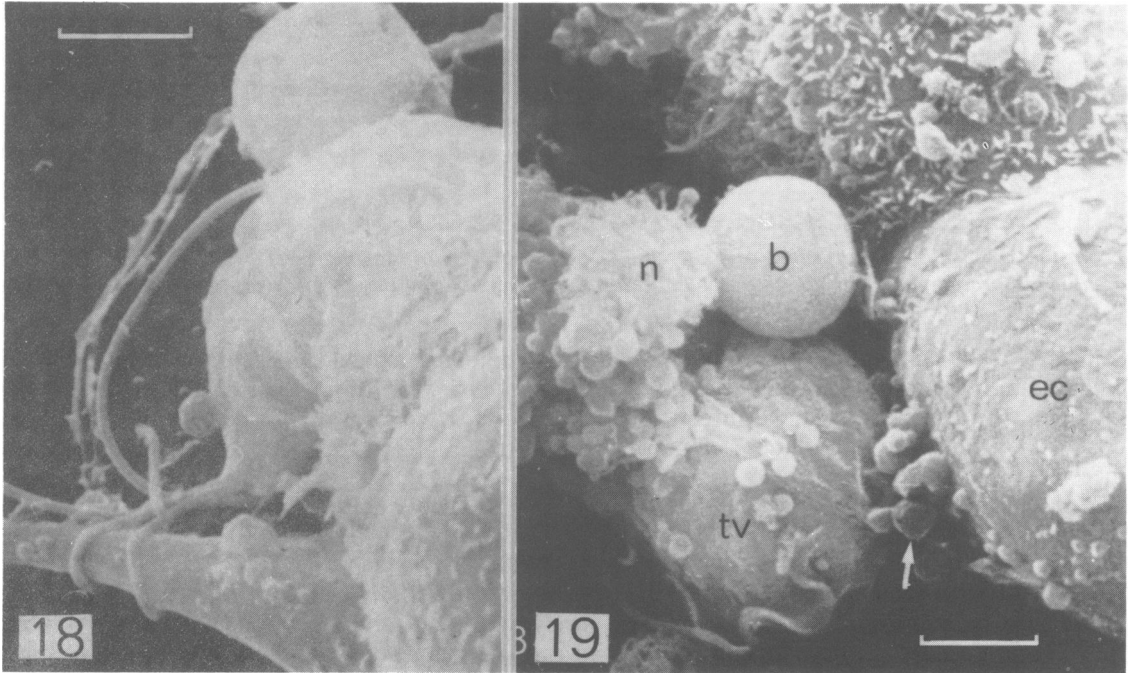30. Lindmark DG, Muller M, Shio H. Hydrogenosomes in *Trichomonas vaginalis. J Parasitol* 1975; 63: 552-4.

FIG 18 *T vaginalis adhering to the side of a rounded epithelial cell. The trichomonad has looped its flagella around a fibre which remained behind as the peripheral parts of the epithelial cell retracted. Scale bar = 2·5 µm*

FIG 19 *Lysis of epithelial cells by T vaginalis. A trichomonad (tv) lies beneath a collection of cytoplasmic constituents, including small vesicles (arrow), the nucleus (n), and bodies (b) of uncertain nature, released by the rupture of the plasma membrane of an epithelial cell. On the right two other epithelial cells are showing signs of injury; both are rounded and the lower cell has lost most of its microvilli. Scale bar = 2·5 µm*