QUICK-FREEZE, DEEP-ETCH ROTARY REPLICATION OF TRYPANOSOMA CRUZI AND HERPETOMONAS MEGASELIAE

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SUMMARY
The fine structure of epimastigotes of Trypanosoma cruzi and promastigotes of Herpetomonas megaseliae was analysed in replicas of quick-frozen, freeze-fractured, deeply etched and rotary-replicated cells. Using control cells and cells treated with Triton X-100 before glutaraldehyde fixation, images were obtained that showed connections of the sub-pellicular microtubules with each other, with the plasma membrane, and with the endoplasmic reticulum. Images were also obtained that showed the DNA network in the kinetoplast. Filamentous structures were found to connect the kinetoplast to the basal body, and to connect the main basal body to the accessory one. In addition, deep-etch images of detergent-extracted flagella display dynein arm substructure and the filamentous architecture of the paraxial structures.

INTRODUCTION
The ultrastructure of trypanosomes, in particular Trypanosoma cruzi, has previously been analysed by transmission electron microscopy of thin-sectioned and freeze-fractured organisms (Martinez-Palomo, de Souza & Gonzales-Robles, 1976; de Souza, Martinez-Palomo & Gonzales-Robles, 1978). In the present paper we extend these reports, by describing the structure of epimastigotes of T. cruzi and promastigotes of Herpetomonas megaseliae as seen in replicas of quick-frozen, freeze-fractured and deeply etched organisms. With this new preparative technique, cells can be frozen directly from life or after brief aldehyde fixation, thus avoiding potentially harmful influences of OsO₄ fixation, chemical dehydration and plastic embedding. In addition, previous studies with this new technique have demonstrated that it provides novel three-dimensional images of membrane surfaces as well as of intracellular filaments and organelles (Heuser & Salpeter, 1979; Heuser & Kirschner, 1980; Hirokawa & Heuser, 1981; Roof & Heuser, 1982). In the present report, we have used this new preparative technique to demonstrate membrane–microtubule interactions in whole cells, and axonemal and kinetoplast architecture in detergent-extracted cells.
MATERIALS AND METHODS

Microorganisms

Epimastigotes of Trypanosoma cruzi, Y strain, were cultivated for 3–4 days at 28 °C in Warren's (1960) medium. Promastigotes of Herpetomonas megaseliae were cultivated in a complex medium (Roitman, Roitman & Azevedo, 1972) for 48 h at 28 °C.

Fixation

The cells were collected by centrifugation and fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h. In some experiments Triton X-100 (final concentration, 0.05–0.1 %) or saponin (0.1 %) was added to the fixative solution.

Quick-freezing, freeze-fracture and deep-etching

Small drops of the pellet containing the parasites were supported on 3 mm × 3 mm × 0.8 mm cushions of rat lung and washed with distilled water containing 15 % (v/v) methanol. Then they were quick-frozen with a liquid helium-cooled copper-block machine (Heuser et al. 1979), and freeze-etched in a Balzer's apparatus as previously described (Heuser & Salpeter, 1979). To ensure that the exposed areas would be optimally frozen, the depth of fracturing was reduced to a minimum: the plane of cleavage entered the sample only a few micrometres beneath its surface and large regions of the surface were not cleaved at all. To limit the depth of exposure around the organelles to 0.1 μm, etching was minimized: the specimen temperature was kept at −105 °C and the etching time was about 2–3 min. Replicas were made by rotary shadowing with a mixture of platinum and carbon, delivered from an electron beam gun mounted at an angle of 24 °. To minimize the thickness of the carbon backing-film, evaporation of carbon was reduced to 3–5 s. Replicas were cleaned in full-strength chromate cleaning solution (Fisher Scientific Co.), washed in distilled water and transferred to 75 mesh grids coated with Formvar plus carbon. Examination was carried out with a Jeol 200CX electron microscope operated at 100 kV. The high accelerating voltage was used to reduce heating of the replica (and hence to minimize recrystallization of platinum), and also to reduce contrast in the final photographic negatives. Stereo pairs were obtained by tilting the sample through ±10 °. Micrographs were examined in negative contrast by photographically reversing them before printing, to make the platinum deposits look white and the background look dark; as previously shown (Heuser et al. 1979), this contrast-reversal enhances the three-dimensional appearance of the images.

RESULTS

The main components of trypanosomes can be identified in deep-etch images such as Fig. 1, which shows a cross-fracture through the central portion of an epimastigote form of T. cruzi. Immediately beneath the plasma membrane are found sub-pellicular microtubules, distributed in the same pattern as is seen in thin sections. What is not apparent in thin sections, however, is that the microtubules lie in a narrow peripheral zone of cytoplasm, which appears to be devoid of the fine granular material that otherwise fills the rest of the cell. This uniformly dispersed granular material, a characteristic feature of all deep-etched cells, is thought to be soluble cytoplasmic protein (Hirokawa & Heuser, 1981; Hirokawa, Tilney, Fujiwara & Heuser, 1982).

With the current technique, images are also obtained of the more traditional fracture faces of the cell membrane; these differ only slightly from our previously published freeze-fracture images (Martinez-Palomé et al. 1976; de Souza et al. 1978), due primarily to the rotary replication used here. This highlights the difference between the high density of P-face particles in the membrane of the cell body and their
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Fig. 1. Survey view of an epimastigote form of *T. cruzi*. Structures such as the nucleus (*n*), nucleolus (*nu*) and the sub-pellicular microtubules (arrows) can be seen. ×31 500.

virtual absence from the membrane of the flagella (Fig. 2 *versus* 3). Larger expanses of fractured membrane such as are shown Fig. 3 illustrate the fact that the particles in the plasma membrane tend to be partitioned into longitudinal clusters that correspond roughly to the spacing of the underlying microtubules. Also the 'cytostome' is readily apparent in fields such as are shown in Fig. 2, where it appears as a region close to the flagellar pocket that contains few particles but is surrounded by a single string of intramembrane particles. Finally, the flagellar membrane itself displays a typical ciliary necklace, plus the occasional group of intramembrane particles, which we have
Figs 2-3
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previously proposed to be involved in the attachment of the flagellum to the cell body (Martinez-Palomo et al. 1976; de Souza et al. 1978).

Returning to the deep-etch images of cell interiors, these typically reveal physical connections between the sub-pellicular microtubules and the plasma membrane (Figs 4, 5). The connections appear to be ~6 nm diameter fibrils that are spaced along the microtubules at intervals of ~20 nm. Similar ~6 nm fibrils are occasionally found to connect the sub-pellicular microtubules to subjacent elements of the endoplasmic reticulum. In addition, images of cells extracted with Triton X-100, in which portions of the plasma membrane are removed, reveal the underlying array of sub-pellicular microtubules particularly clearly (Fig. 6). In such images, similar 5–6 nm fibrils are also found to connect the microtubules to each other (at intervals of 12 to 30 nm).

Triton-extracted cells also provide numerous images of flagellar axonemes that display their outer dynein arms quite distinctly (Fig. 7). These images display sub-structure in the individual dynein arm (a bulky head attached to one A-microtubule and a slender 'stalk' reaching over to the B-microtubule of the adjacent doublet) that differs only in minor details from the substructure of outer dynein arms in other protzoa, as described by Goodenough & Heuser (1982). As in other protozoa, adjacent dynein arms are spaced ~24 nm apart and appear to be linked to each other by diagonal components termed 'linkers' by Goodenough & Heuser (1982). (See Goodenough & Heuser (1984) for an explanation for the piggyback-overlap of dynein molecules that gives rise to this image.)

Also visible in deep-etch images of detergent-extracted flagella is the characteristic 'paraxial structure'. As was apparent in thin sections, this structure is composed of a complex fabric of ~8 nm filaments woven together by narrower oblique elements. Particularly distinct in deep-etch replicas are the numerous short projections that connect the paraxial structure with the B-microtubules of the adjacent axoneme.

The kinetoplast can also be easily recognized in deep-etch images. In non-extracted cells, in which the mitochondrial membrane is intact, its core of kinetoplast DNA looks like a dense rod that is opposed to the portion of the mitochondrial membrane located near the nucleus. This rod is connected by thin filamentous structures to the opposite side of the mitochondrion, where its membrane approaches the basal body (data not shown). In Triton X-100-extracted cells, where membranes are largely extracted and the granular material of the cytoplasm is removed so that structures like the kinetoplast can be seen to advantage, it is apparent that the kinetoplast DNA filaments are woven together to form radially oriented bundles. These are connected to each other laterally by thinner filaments that appear to branch and anastomose to create polygonal 'facets'. In the periphery of the kinetoplast, these filaments appear to extend between the thicker bundles of DNA and remnants of the mitochondrial.
membrane (which usually persist only on the basal body side). In addition, stouter filaments (11 nm in diameter) connect the remains of the mitochondrial membrane to the two basal bodies, one of which is always associated with the flagellum (Figs 8–9). Filamentous structures also connect the basal body at the base of the flagellum to a second, lateral or accessory basal body (Fig. 9).

Figs 4–5. Cross-sections of epimastigotes of T. cruzi. The arrows in Fig. 4 indicate fibrils connecting the sub-pellicular microtubules to the endoplasmic reticulum (er, indicated by a dense line). The arrows in Fig. 5 indicate connections of the sub-pellicular microtubules to the plasma membrane. ×130000.

Fig. 6. Promastigotes of H. megaseliae treated with Triton X-100 immediately before glutaraldehyde fixation. The plasma membrane removed, revealing the underlying layer of sub-pellicular microtubules (mt) arranged in a parallel array. Fibrils can be seen connecting the sub-pellicular microtubules to each other (arrow). ×170000.
DISCUSSION

Previous electron microscopy of *T. cruzi* has depended primarily upon preparation of thin sections of chemically fixed cells. This traditional approach entails some potential problems. Actin filaments, if not associated with other proteins, can break down during osmium tetroxide fixation (Maupin-Zamier & Pollard, 1978) or during chemical dehydration (Small, 1982). Also, plastic embedding can create a background electron density that obscures the finer filamentous structures of the cytoplasm (Wolosewick & Porter, 1976). Trypanosomes have also been prepared for electron microscopy by freeze-fracture, a method that yields particular information on the internal organization of their membranes. Freeze-fracture, however, cannot provide additional information on internal structures such as the kinetoplast or the axoneme-paraxial assembly inside flagella. In contrast, the deep-etch technique used here offers the advantage that it allows simultaneous examination of the fracture faces and the true surfaces of various membranes, as well as the topography of intracellular structures that have not been fixed with osmium tetroxide or dehydrated with chemicals.

Our results with this technique essentially confirm previous freeze-fracture studies on various organisms (*T. cruzi*: Martinez-Palomo *et al.* 1976; de Souza *et al.* 1978; *T. brucei*: Smith, Njogu, Cayer & Jarlfor, 1974; Hogan & Patton, 1976; Vickerman & Tettley, 1977; *Leptomonas collosoma*: Linder & Staehelin, 1977; *Herpetomonas samuelpessoai*: de Souza, Chaves & Martinez-Palomo, 1979; *Leptomonas samueli*: Souto-Padrón, Gonçalves de Lima, Roitman & de Souza, 1980; and *Leishmania mexicana amazonensis*: Benchimol & de Souza, 1980). In addition, the deep-etch images presented here provide new information on internal structures such as the subpellicular microtubules, the flagellar axoneme and the kinetoplast–basal body assembly.

The flagellum

Previous thin-section studies have shown that axonemes in *T. cruzi* flagella have the typical 9+2 pattern of microtubules, but these studies have not provided any clear images of dynein arms or radial spokes. In contrast, the present study illustrates the fact that when an axoneme happens to lie parallel or slightly obliquely to the fracture plane, but slightly beneath it, deep-etching will reveal its surface structure very...
clearly. This has shown that the architecture of the outer dynein arms in *T. cruzi* axonemes conforms closely to the description given by Goodenough & Heuser (1982) for other protozoa. (That study should be consulted for details.)

The sub-pellicular microtubules

A characteristic feature of trypanosomes is a layer of microtubules beneath their plasma membrane. These microtubules remain attached to the plasma membrane and to each other when cells are ruptured and their plasma membranes isolated (Hunt & Ellar, 1974; de Souza, 1976; Linder & Staehelin, 1977; Voorheis, Gale, Owen & Edwards, 1978; Dwyer, 1980), indicating they are indeed attached to the plasma membrane. Previous thin-section and negative-stain studies have shown that these attachments take the form of microtubule side-arms that recur with a periodicity of 22–24 nm and extend towards the plasma membrane as either single entities or triplets that fan out laterally to attach to the membrane. In our deep-etch images of microtubule–membrane crossfractures, we also see two or three projections fanning out laterally from each microtubule to reach the adjacent membrane. These small 'fans' recur at ~20 nm in our replicas. The exact mode of their attachment to the plasma membrane remains to be seen, but in this regard it should be pointed out that the non-random deployment of intramembrane particles and lipids seen in the plasma membrane of these organisms (Souto-Padrón & de Souza, 1983) most probably reflects the patterning of the underlying lattice of microtubules.

The chemical nature of these microtubule–membrane bridges remains unknown, but the present observations tend to discount Dwyer's (1980) suggestion that they might be dynein. This is because dynein has such a distinct substructure when seen by deep-etching (Fig. 7) while the side-arms in question look completely different (Figs 4–6). The latter are much thinner overall, and lack any of the globular expansions of dynein. We are more impressed with their structural similarity to deep-etched microtubule-associated proteins (Heuser & Kirschner, 1980).

The kinetoplast

Previous thin-section studies of trypanosomes have demonstrated that the kinetoplast of epimastigotes consists of a complex array of delicate filaments (2-5 nm in diameter), which here and there contact the inner mitochondrial membrane (reviewed by Vickerman & Preston, 1976). Electron microscopy of the DNA in the kinetoplast, isolated after lysis of the cell, indicates that it consists of a network of 20 000 to 30 000 'minicircles', each with a length of ~0.45 μm, plus some larger circles (called 'maxicircles') each with a length of about 10 μm (reviewed by Borst & Hoeijmakers, 1979). In the present study, deep-etching has permitted three-dimensional examination of the kinetoplast–DNA network in intact cells. We find that the DNA is packed into dense bundles that belie its underlying arrangement into circles. Presumably, this underlying structure becomes apparent only during isolation, when the DNA may lose some components involved in its *in situ* packing. Unfortunately, the images provided here offer no ready explanation as to why maxicircles are seen preferentially at the periphery of spread DNA preparations (cf. fig. 2 of Hadjuk &
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Cosgrove, 1979; figs 1–6 of Hoijmakers & Weijers, 1980; fig. 1 of Borst & Hoeijmakers, 1979; and fig. 3 of Leon et al. 1980).

Kinetoplast–basal body association

During the normal life-cycle of trypanosomes, the position of the kinetoplast relative to the nucleus changes; in the amastigote form the kinetoplast is located in the anterior portion of the cell, while in the trypomastigote form it is located in the posterior region. In both cases, however, the kinetoplast is located close to the basal body. Although previous ultrastructural studies have not shown any structural links between the two, they also tend to hold together during isolation (Braly, Simpson & Kretzen, 1977), except when divalent cations are removed (Simpson, 1972). The deep-etch images presented here illustrate the fact that the structural entities maintaining this connection are relatively stout fibrils, ~11 nm in diameter. Their chemical nature remains to be determined.

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REFERENCES


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