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An Electron Microscopic and Cytochemical Study of the Cell Coat of *Trypanosoma cruzi* in Tissue Cultures*

Wanderley De Souza and H. Meyer

Instituto de Biofisica-UFRJ, Cidade Universitária Rio de Janeiro

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Summary. Using the cytochemical method of Thiery, a polysaccharide surface coat was demonstrated with the electron microscope in the various developmental stages of *Trypanosoma cruzi* maintained in tissue cultures. The cell coat was observed on the whole surface membrane system of *T. cruzi.* A positive reaction, similar to that observed in the cell membrane, was also obtained in the membranes of some vacuoles in the cytoplasm of the parasites. No granules were found in the parasite which could represent reserve polysaccharides.

The possible role of the cell coat in such phenomena as adhesion, agglutination, phago- and pinocytosis and antigenicity is discussed.

Introduction

Much evidence has been presented for the existence of carbohydrates in the cell membrane, and that these take part in the formation of the cell coat.

Recently, investigations have been carried out to study the cell coat of trypanosomatids. Wright and Hales (1970) using histochemical methods examined trypomastigotes from the blood of rats infected with *Trypanosoma brucei*. Dwyer (1974), Dwyer and D'Alesandro (1974) and Dwyer *et al.* (1974) studied promastigotes of *Leishmania donovani* from an acellular culture medium and blood trypomastigotes of *Trypanosoma duttoni*, using histochemical methods and agglutination by lectins.

In order to study the cell coat of *Trypanosoma cruzi* we began a series of cytochemical investigations on tissue culture forms, the results of which will be given in this paper. They show the reaction of all the intra- and extracellular forms, occuring in tissue cultures, to the application of the method of Thiery (1967).

Materials and Methods

The *Trypanosoma cruzi* used in this study were maintained in tissue cultures of embryonic chick heart muscle cells. They were isolated from the blood of a patient in the state of Goiás, Brazil and have been maintained for more than 15 years in tissue cultures with the methods used in this laboratory and previously described (Meyer and Xavier de Oliveira, 1948). When the cultures showed cells with parasites in the desired stage, they were fixed whole with 2.5% (v/v) glutaraldehyde for 30 min and after, with 1% (w/v) OsO^4 for 1 hr in the refrigerator. Both fixatives were diluted with the same Tyrode solution that had been

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used for the culturing of the cells (Meyer, 1968). Dehydration was effected in acetone and then, the cultures were cut into small pieces, removed from the cover slips and stained with 0.3% (w/v) uranyl acetate in acetone overnight, and after thorough washing in acetone they were embedded in Epon resin. Ultrathin sections were cut on a LKB Ultratome III Ultramicrotome displaying light-gold to silver interference colors, were collected on copper grids and examined unstained or stained with lead citrate in an AEI EM6-B electron microscope with a 50 μ objective aperture and operating at 60 KV.

For the cytochemical experiments, ultrathin sections were collected on gold grids and stained by the periodic acid-thiosemicarbazide-silver proteinate technique as recommended by Thiery (1967). In this method ultrathin sections are first oxidized by periodic acid to convert adjacent hydroxyl or α-amino alcohol groups into aldehydes. These aldehydes are then condensed with thiosemicarbazide to yield thiosemicarbazones which are powerful reducing agents. Thus after exposure to silver proteinate these thiosemicarbazones are finally revealed in the electron microscope as silver deposits at the reactive sites. Control sections were treated similarly to these experimental sections except for the omission of one of the steps.

Results

The ultrastructure of the various stages in the life cycle of *T. cruzi* in tissue cultures has been described before (Meyer *et al.*, 1958; Meyer, 1968, 1969; Meyer and De Souza, 1973; De Souza and Meyer, 1974). The membrane of the parasite shows the characteristic tri-laminar structure, with a thickness of about 80 Å and below this membrane a row of microtubules (Fig. 1). In preparations which are stained with uranyl acetate and lead citrate no surface coat is observed like that found in *T. brucei* (Vickerman, 1969; Wright and Hales, 1970).

Using the method of Thiery, however, a positive reaction is found in the form of an electron-dense deposit on the external face of the cell membrane which gives it an asymmetric aspect (Fig. 3, 6). This reaction is observed on the whole surface membrane system of *T. cruzi*, e.g. on the membrane which covers the body of the parasite (Figs. 3, 6), the region of the flagellar pocket and the flagellum (Figs. 4, 5). It is found in all the tissue culture forms, in the extracellular trypomastigotes as well as in the intracellular amastigotes, epimastigotes and trypomastigotes. The intensity of the reaction is the same in all the membranes; it is more pronounced when the incubation time in thiosemicarbazide is prolonged (Figs. 3, 6).

In the interior of the parasite vacuoles may be found near the flagellar pocket, the Golgi complex, and in the posterior region. There is a positive reaction in the membranes of many of these vacuoles, similar to that observed in the surface membrane (Fig. 7). No positive reactions were found in the other internal membrane systems such as the Golgi zone, the endoplasmic reticulum, or the kinetoplast-mitochondria complex. No cytoplasmic granules have been found in the parasite which could represent reserve polysaccharides, such as those seen in other protozoa.

In the various controls, which we examined, no reactions have been found in the structures of the parasite. The preparations looked like unstained sections, showed no contrast at all and were difficult to focus with the electron microscope (Fig. 2).

Discussion

With the introduction of cytochemical techniques for the detection of carbohydrates the occurrence of a cell coat was described which did not appear when

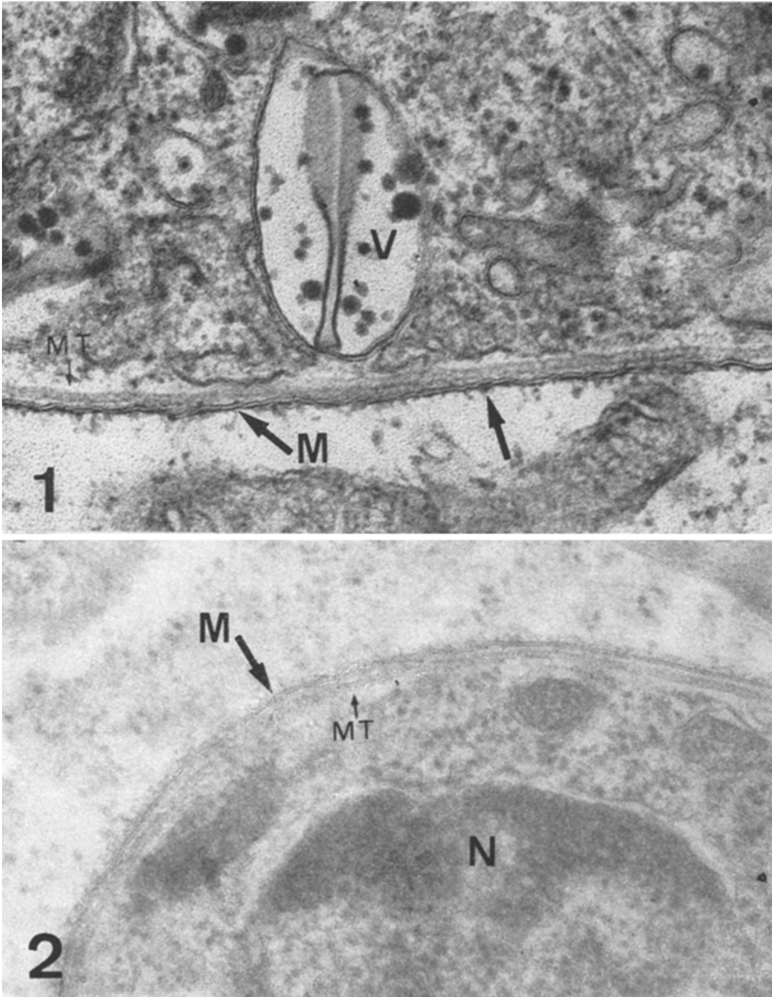

Fig. 1. *Trypanosoma cruzi.* Part of an intracellular amastigote stained with lead citrate. The tri-laminar structure of the cell membrane (*M*) is evident. A dense material can be observed outside the cell membrane (arrow). A sub-pellicular microtubule (*MT*) and a vacuole (*V*) limited by a membrane is also observed in the interior of the parasite. × 75000

Fig. 2. *Trypanosoma cruzi.* Part of an intracellular amastigote not stained with lead citrate. The section was oxidized by periodic acid and incubated directly in silver proteinate without previous incubation in thiosemicarbazide. The structures show little contrast. Note the cell membrane (*M*), sub-pellicular microtubule (*MT*) and nucleus (*N*). ×60000

the cells were observed by the usual electron microscopic methods (Bennet, 1963; Rambourg and Leblond, 1967; Martinez-Palomo, 1970; Rambourg, 1970; Schrével, 1972; Parson and Subjeck, 1972; Wyroba and Przellecka, 1973; Seed *et al.*, 1974).

Among the cytochemical methods used the most important were those in which cationic stains were employed which are able to bind with anionic groups,

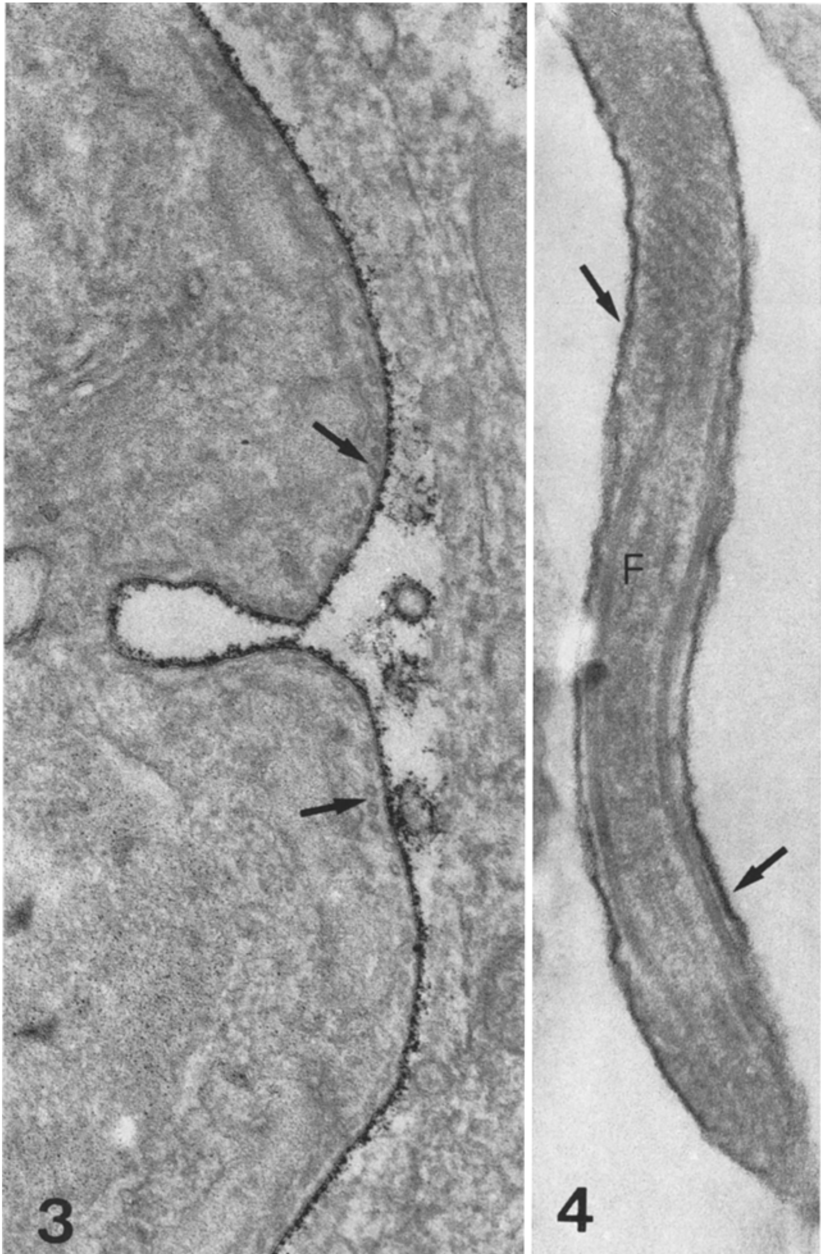

Fig. 3. *Trypanosoma cruzi*. Part of an intracellular amastigote during division, showing invagination of the cell membrane. The section was incubated for 76 hrs in thiosemicarbazide. The reaction shows a dense material localized on the outer face of the cell membrane. A sub-pellicular row of microtubules (arrows) is observed. $\times 45000$

Fig. 4. *Trypanosoma cruzi*. Flagellum (*F*) from an extracellular trypomastigote. Preparation incubated for $2^1/_2$ hrs in thiosemicarbazide. Note the dense substance localized at the periphery of the flagellum (arrow). $\times 60000$

as for instance colloidal iron and thorium (Rambourg and Leblond, 1967; Gasic *et al.*, 1968), ruthenium red (Luft, 1971) and Alcian blue associated with lanthanum nitrate (Shea, 1971). However, these substances do not penetrate into the cells, but remain on the surface only, and can therefore not be applied for the study of intracellular parasites. Other techniques exist which are adaptations of the PAS for electron microscopy. Among these, Thiery's (1967) is the most used.

Vickerman (1969) showed in various trypanosome strains ultrastructural modifications at the cell surface. Trypanosomes in the final form of their cycle in the insect vector show a thick coat which is absent, however, in the proliferative forms in the insect as well as in an acellular medium. This coat is sensitive to the action of pronase and negative to ruthenium red which led Vickerman to assume that it was composed of proteic material.

Strauss (1971) showed in *Leishmania tarentolae*, in which normally no coat is observed, that one develops when the parasites are cultured in a medium to which homologous rabbit antiserum is added. Wright and Hales (1970) showed that the dimensions of the coat in *T. brucei* vary according to the type of fixation used. These coats reacted positively to the method of Thiery as well as with ruthenium red.

However, all these investigations have been done on free forms of trypanosomes e.g. blood forms or forms from acellular culture medium. Dwyer *et al.* (1974) isolated amastigotes of *L. donovani* by centrifugation methods and cultured them in an acellular medium. They waited for their transformation *in vitro* into the promastigote forms. The cell coat was demonstrated after 3 hrs of cultivation, the time necessary for the parasite to lose the membrane which comes from the host cell and surrounds the intracellular parasites. In those amastigotes, however, which had lost this membrane during the isolation procedure the reaction was positive immediately, which suggests that the cell coat exists also in the intracellular forms of *L. donovani*.

The positive reaction obtained with our material using the method of Thiery, on the whole surface membrane system of *T. cruzi*, shows that carbohydrates constituents exist in the cell coat of *T. cruzi*, both in the membrane which covers the body, and in the flagellar pocket and flagellum of all the intracellular forms of the parasite.

In the trypanosomatids, as in other cells, the cell coat possibly plays an important role in such phenomena as adhesion, agglutination, phago- and pinocytosis and antigenicity. As to adhesion, there is the tendency to form rosettes *in vitro*, the adherence of the parasites in the alimentary tract of the insect hosts (Molyneux, 1969; Vickerman, 1973) and the adhesion of the flagellum to the body of the parasite, especially evident in the trypomastigote forms (Meyer, 1968, 1969; Vickerman, 1969). As to phagocytosis and pinocytosis, it is known that the trypanosomes are able to ingest material by pinocytosis in the region of the flagellar pocket (Brown *et al.*, 1965; Milder and Deane, 1969) or by phagocytosis by a process of intracellular phagotrophy (Meyer and De Souza, 1973). In these processes the cell coat, due to its capacity of combining with certain chemical groups, might select the material to be ingested. With regard to the antigenic capacity, Vickerman (1969) and Vickerman and Luckins (1969) suggested that agglutinogens are located in the coat of *T. brucei*.

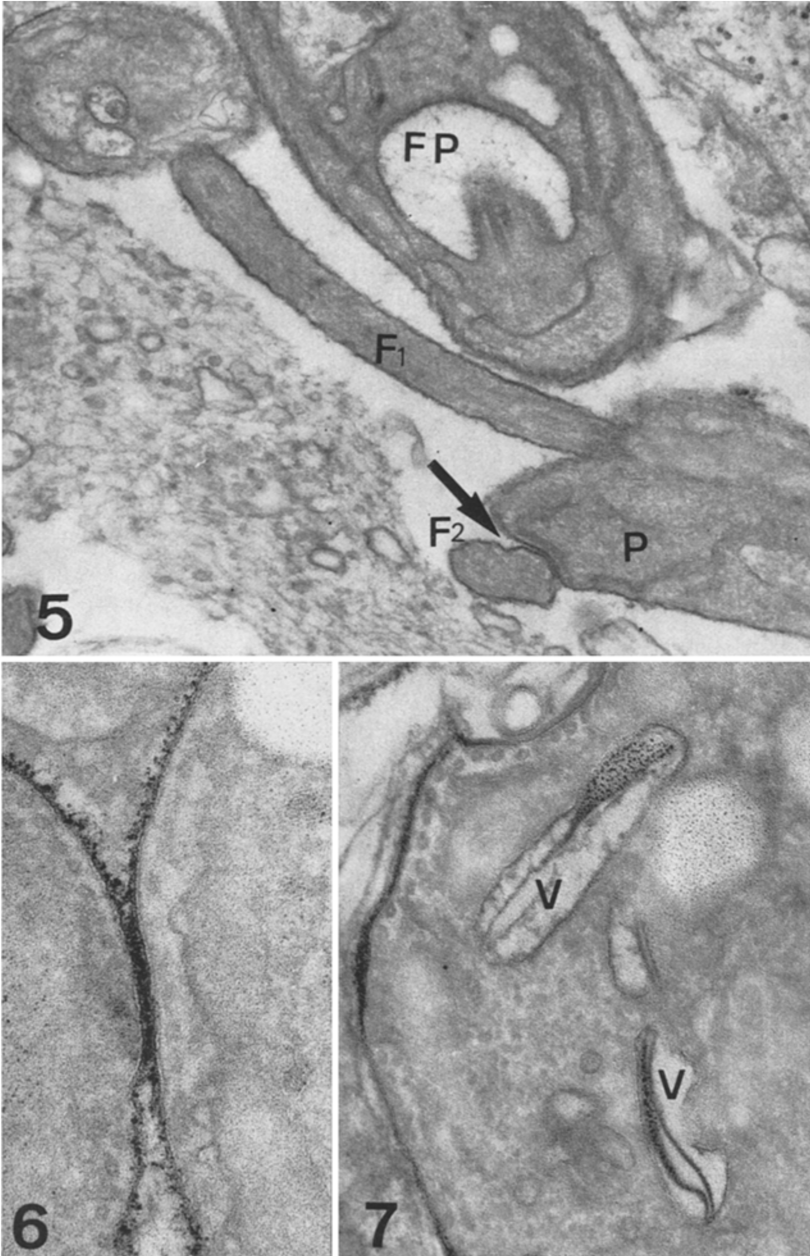

Fig. 5. *Trypanosoma cruzi.* Parts of parasites during intracellular transformation from amastigote to trypomastigote. Preparation incubated for $2^1/_2$ hrs in thiosemicarbazide. Note the dense material on the whole surface of the flagellum (*F1*), in the flagellar pocket (*FP*) and in the attachment region (arrow) of the flagellum (*F2*) to the body of the parasite (*P*). $\times$ 30000

It was shown recently that trypanosomatids agglutinate, when treated with lectins, substances which are specific to detect terminal residues of polysaccharides and glycoproteins on cell surfaces. Thus Dwyer (1974) showed agglutination of promastigotes of *L. donovani* with concanavalin A and phytohemagglutinins M and P. A similar result was obtained with blood forms of *T. duttoni* with concanavalin A (Dwyer and D'Alesandro, 1974). In all these studies the cell coat was also observed by cytochemical methods. Alves and Colli (1974) showed that epimastigotes of *T. cruzi* from acellular cultures agglutinate in the presence of concanavalin A, which does not occur with the trypomastigotes of the blood. The agglutination was not affected by tripsinisation, but was inhibited by α-d-mannose, α-d-glucose and N-acetyl d-glucosamine.

Moura Gonçalves and Yamaha (1959) identified by chromatographic analysis of hydrolysates of *T. cruzi* from acellular cultures the presence of a polysaccharidic fraction in which it was possible to demonstrate the presence of glucose, glucosamine, xylose, mannose and galactose. The sample was considered a polysaccharidic-polypeptidic complex which showed immunological activity. It is possible that this polysaccharidic-polypeptidic fraction corresponds to the polysaccharides and glycoproteins in the membrane of *T. cruzi*, observed by us and described in this paper.

Vacuoles such as those which are found in the interior of *T. bruci* (Wright and Hales, 1970), limited by a membrane which gives a positive reaction, have also been found in all the forms of *T. cruzi*. They probably represent vacuoles formed by pinocytosis and phagocytosis.

Biochemical studies on the intracellular forms of *T. cruzi* are practically nonexistent. All the data which we were able to find refer to the epimastigote forms obtained in an acellular medium (von Brand, 1966). In these no reserve polysaccharides were found. In our tissue culture material we did not find structures in the cytoplasm of the parasite which could represent reserve polysaccharides as observed in other protozoan cells (Ryley *et al.*, 1969; Schrevel, 1970; Sénaud *et al.*, 1972).

Key to Letterings

F	flagellum	N	nucleus
FP	flagellar pocket	P	parasite
M	cell membrane	V	vacuole
MT	sub-pellicular microtubule		

Fig. 6. *Trypanosoma cruzi*. Part of two intracellular amastigotes. Preparation incubated for 76 hrs in thiosemicarbazide. Note the dense material localized on the outer surface of the parasite membrane, specially evident in the region of contact of the two parasites. ×75000

Fig. 7. *Trypanosoma cruzi*. Part of an intracellular amastigote. Preparation incubated for $2^1/_2$ hrs in thiosemicarbazide. Note the presence of vacuoles (*V*) in the cytoplasm of the parasite, limited by a membrane with a positive reaction similar to that observed in the cell membrane. ×60000

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Dr. Wanderley De Souza
Instituto de Biofísica—UFRJ
Cidade Universitária
Rio de Janeiro-Gb., Brasil