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IMMUNOHISTOCHEMICAL STAINING OF TRYPANOSOMA CRUZI CELL MEMBRANE. LOSS OF ANTIGENIC DETERMINANTS FOLLOWING SALINE EXTRACTION

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SUMMARY

By immunohistochemical methods employing rabbit anti-**T. cruzi** serum conjugated to horseradish peroxidase developed by a suitable substrate, it was possible to identify the presence of two types of antigenic determinants on the membrane of **T. cruzi** (Y strain) culture forms. Following saline extraction for various periods of time such determinants were removed from the flagellate's surface. Immunofluorescence tests performed using saline extracted epimastigotes as antigen showed that extraction by phosphate-buffered saline removed the receptors for such tests from the epimastigotes membrane surface.

INTRODUCTION

Antigenic determinants have been described by immunohistochemical techniques on the surface of the **T. cruzi** (MILDER & GUIMARÃES, 1976; FRUIT et al., 1978). The electron dense product was distributed throughout the parasites surface: at the plasma membrane, cytostome, flagellar pocket and flagellum (MILDER & GUIMARÃES, 1976).

In 1977 GUIMARÃES et al. reported that following saline extraction **T. cruzi** epimastigotes lost the antigenic determinants responsible for immunofluorescence tests. Upon subculture the flagellates were able to resynthetize to a great extent, the antigenic determinants lost during extraction (GUIMARÃES & RIBEIRO, 1978b).

The present paper reports the localization of such determinants at the ultrastructural level of non extracted cells, of saline or trypsin extracted epimastigotes and correlates such findings with immunofluorescence results.

MATERIAL AND METHODS

Y strain epimastigotes (SILVA & NUSSENZVEIG, 1953) grown in LIT medium (CAMARGO, 1964) were used throughout this study. Two days old flagellates were washed 5 times in PBS (phosphate buffered saline 0.5 M, pH 7.2, 0.01 M in phosphate) and were used either without any previous treatment or subsequent to saline extraction or trypsinization.

Saline extraction was carried out with PBS for 6, 24 or 72 hours as previously reported (GUIMARÃES et. al., 1977) and trypsinization was carried out in 5% trypsin at 37°C for 60 min. (VATTUONE & YANOVSKY, 1971).

Electron microscopy

Parasites without treatment or after trypsinization were spun down at 1.500 G, washed 3 times in PBS and fixed with either 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3 for 30 min. at room temperature or in PLP (periodate-lysine-paraformaldehyde) in 0.0375 M

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phosphate buffer, pH 7.4 (WILSON-KIMBALL & NAKANE, 1978). Extracted cells were fixed in 2% glutaraldehyde as described above.

After fixation cells were washed 3 times in cacodylate buffer (0.2 M, pH 7.2) spun down at 1.500 g and submitted to a direct immunoperoxidase test as follows: the cell pellet was gently suspended in a 1:5 dilution of horseradish peroxidase labelled rabbit anti-**T. cruzi** serum and incubated for 2 hours at 37°C and enzyme activity developed by 3,3'-diaminobenzidine (GRAHAM & KARNOVSKY, 1966).

The cells were then washed 2 times in cacodylate buffer and postfixed in 1% buffered OsO4 for 1 hour. Dehydration was carried out in a graded ethanol series, treated with propilene oxide and embedded in Araldite. Thin sections were examined in a Zeiss EM 9S electron microscope, unstained or briefly stained with uranyl acetate.

Normal rabbit serum conjugated to horseradish peroxidase was used as control.

Preparation of conjugate

20 mg of type VI horseradish peroxidase (Sigma Chemical Co.) in carbonate buffer (0.3M, pH 8.1) were added to 20 mg of rabbit anti-**T. cruzi** serum. The conjugate was prepared according to NAKANE & KAWAOI, 1974. Protein was assayed by the biuret method (GORNALL et al., 1949) and peroxidase content by radial immunodiffusion (GUIMARÃES et al., 1978a). The molecular ratio of enzyme: total protein was 0.034 (protein content 1.0 mg/ml); horseradish peroxidase 0.12 mg/ml).

The antiserum was raised in rabbits immunized with three doses of 2 mg of freeze dried Y strain epimastigotes, one week apart. The first one was emulsified in Complete Freund's Adjuvant, the second in incomplete Freund's adjuvant, both subcutaneously and the third was injected intra-venously. At the 4th week the rabbits were bled from the heart, the blood allowed to clot and serum aliquots kept at -70°C.

Immunodiffusion

ID was carried out on 0.9% agarose in 0.15 M saline. Peripheral wells contained a crude extract from freeze-dried epimastigotes, the supernatant from trypsin extracted epimastigotes and the supernatant from 24 hours PBS extracted epimastigotes. The center well contained a rabbit anti-**T. cruzi** serum, the same one used for conjugation to peroxidase. Diffusion proceeded for 24 hours in a moist chamber at room temperature and after washing the gel was dried and stained with Light Green (URIEL, 1971).

Immunofluorescence

The indirect immunofluorescence test was carried out according to CAMARGO, 1966. As antigen, either PBS extracted, trypsin extracted epimastigotes or non-extracted epimastigotes were used (GUIMARÃES et al., 1977). The sera came from Chagas'disease patients possessing either IgG or IgM immunoglobulins, at doubling dilutions, maintained in this laboratory as standards. The conjugates were either FITC-rabbit anti-human gamma chain or FITC-rabbit anti-human μ chain (Travenol Laboratories) and had an F/P ratio of 3.3 and 2.1 respectively.

Immunofluorescence blocking — The same standard sera at doubling dilutions (starting dilution 1/10) were mixed v/v with PBS extracted antigen at a protein concentration of 80 micrograms/ml and left overnight at 4°C. The indirect immunofluorescence test that followed was carried out as indicated above.

RESULTS

Non-extracted cells — In spite of the several steps involved in immunohistochemical staining, non-extracted cells fixed by glutaraldehyde appeared well preserved.

There was a very poor fixation of epimastigotes by PLP with cells displaying many signs of damage (such as poor organelle preservation, intense cytoplasma vacuolization and loss of nuclei membrane integrity).

The deposition of electron-dense material on non-extracted cells fixed by glutaraldehyde showed 2 staining patterns one, "linear" alongside the cytoplasmic membrane (arrow, Fig. 1 and 1a) and another as globular aggregates (hatched arrow, Fig. 1 and 1a). Both were deposited throughout the entire surface of the parasite: at the plasma membrane, flagellar pocket and flagellum. Metacyclic trypomastigotes displayed the same staining pattern as epimastigotes (Fig. 3) although apparently the globular

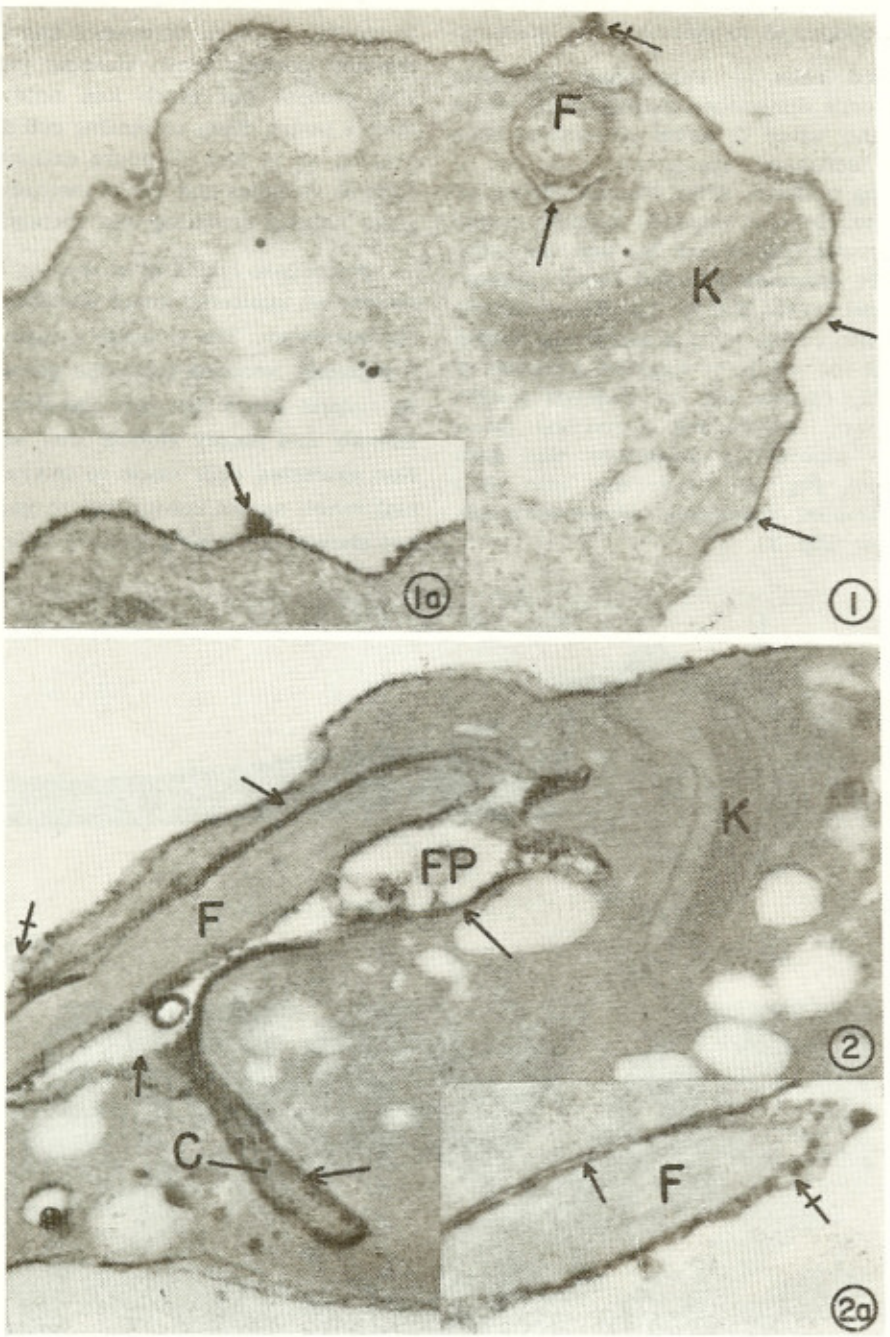


Fig. 1 — Non-extracted epimastigote. Arrows-linear staining; hatched arrows-globular aggregates; F-flagellum; K-kinetoplast. The conjugate is deposited at the cell membrane, flagellar pocket and flagellum. 36,000 ×. Fig. 1a — Large magnification of the globular aggregate. 47,500 ×.

Fig. 2 — 6 hours extraction. Arrow-linear staining; hatched arrow-empty globular aggregate; short arrow-amorphous substance; F-flagellum; FP-flagellar pocket; K-kinetoplast; C-cytostome. The linear staining pattern is present at this extraction period. The globular aggregates have became empty. 38,000 ×. Fig. 2a — 6 hours extraction. Arrow-the linear deposit at the region of flagellar adhesion to cell body; hatched arrow-empty globular aggregates. 47,500 ×.

aggregate was less prominent than the linear one, when compared to epimastigotes staining.

Extracted cells — Peroxidase conjugate binding to cells diminished as extraction times increased and, after 72 hours extraction, cells assumed a "scrubbed" appearence with loss of both staining patterns. After 6 hours extraction many of the globular deposits became empty (hatched arrows, Fig. 2 and 2a) and the parasite's surface assumed a "moth eaten" appearence (hatched arrow, Fig. 2). The linear deposit was still quite visible at this extraction period especially at the region of flagellar adhesion to the cell body (arrow, Fig. 2), flagellar pocket and cytostome (arrows, Fig. 2 and 2a). Occasionally an amorphous substance was seen (short arrow, Fig. 2) which was also seen in non extracted metacyclic trypomastigotes (short arrow, Fig. 3).

After 24 hours extraction there was an intermediate picture between 6 and 72 hours extraction periods. Both staining patterns were still present but much less noteworthy than after 6 hours. Signs indicating cell damage were present at 24 and 72 hours extraction periods such as vacuoles and nuclei membrane irregularities namely crinkling and retraction (Fig. 4).

Peroxidase labelling of trypsin treated cells showed an unaltered linear labelling of the plasma membrane. The globular antigen was almost completely removed from the parasites surface by trypsin extraction and therefore only occasionally and empty globule was seen (Fig. 5). Non extracted cells made to interact with normal rabbit serum conjugated to peroxidase did not show any staining pattern (Fig. 6).

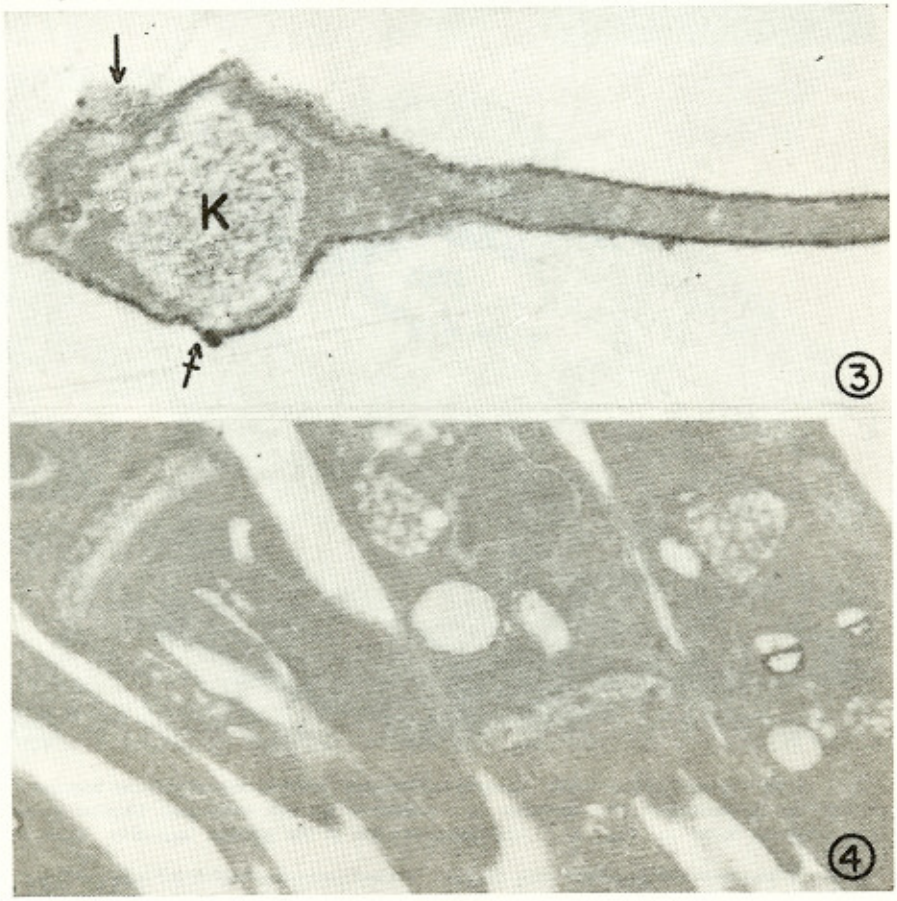
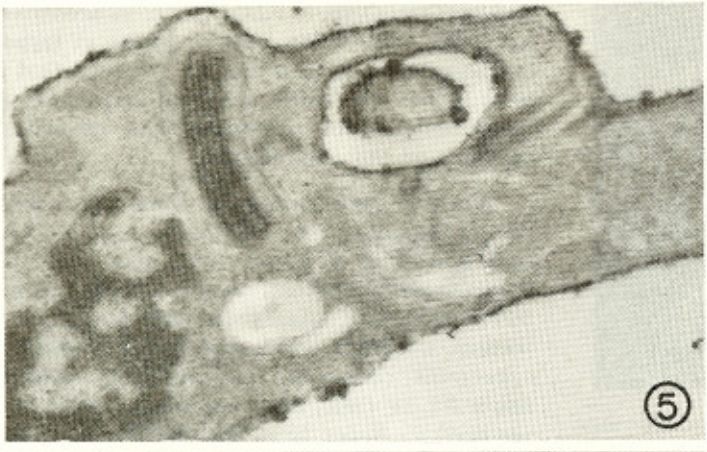


Fig. 3 — Metacyclic trypomastigotes. Short arrow-amorphous substance; hatched arrow-globular aggregate. The linear staining pattern is present at the parasite's cell membrane. 36,000 ×.
Fig. 4 — 72 hours extraction — Disappearence of both staining patterns. 28,500 ×



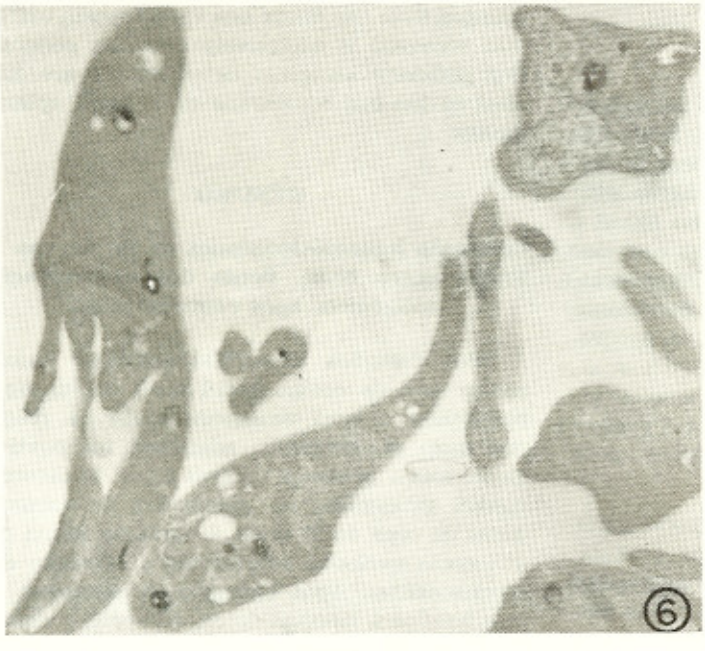


Fig. 5 — Trypsin extraction. Partial removal of the globular aggregate. The linear deposit is present. 36,000 ×.
Fig. 6 — Control reaction. Absence of either staining pattern. 18,000 ×.

Immunodiffusion — As seen in Fig. 7 the 3 supernatants shared one identical band.

Immunofluorescence — IgG (titer range 1/320 to 1/640) or IgM standard sera (titer range 1/80 to 1/160) became negative when PBS-extracted epimastigotes were used or when blocking of immunofluorescence was induced by previously mixing Chagas'human standard sera to PBS-supernatant. Sera titers fell to 70% of their previous values when trypsin extraction epimastigotes were used as antigen fixed to microscope slides.

DISCUSSION

Glutaraldehyde proved to be a better fixative for epimastigotes regarding electron microscopy results than PLP. Very poor fixation was obtained by the latter method and cells very badly preserved.

Deposition of conjugate on the epimastigote membrane was uniform throughout the whole cell surface over the cytostome, flagellar and flagellum for non-extracted cells. This fact points out the similarity between those spe-

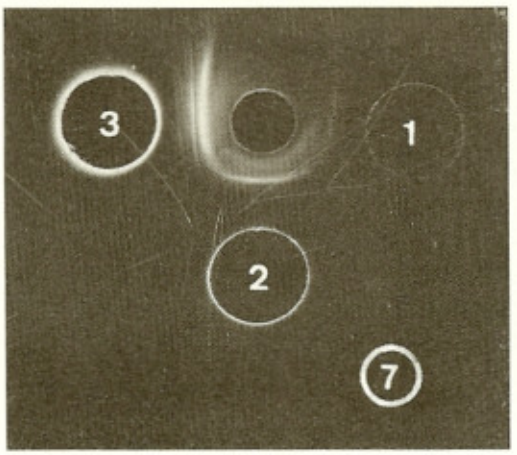


Fig. 7 — Immunodiffusion of supernatants. Center well-rabbit anti-**T. cruzi** serum. Peripheral wells: 1 — supernatant of PBS extraction; 2 — supernatant of trypsin extraction; 3 — crude **T. cruzi** extract.

cialized segments for the membrane as already shown by MILDER & GUIMARÃES, 1976. The same pattern was shown to be present in trypomastigotes. These findings do not agree with MARTINEZ-PALOMO et al., 1976, who found a prominent specialized surface coat at one side of the cytostome region of **T. cruzi** demonstrated by a heavier labeling with ConA peroxidase and ruthenium red. Our findings are in accordance with those reported by MEHLHORN et al., 1977, who described in cell culture epimastigotes a thin surface coat which was also present within the flagellar pocket and along the inside of the cytostome.

PBS extraction of live epimastigotes resulted in disappearence of the staining pattern; after 6 hours extraction the globular aggregates became empty but the linear staining was present at the parasite's surface and also at the region of flagellar adhesion to cell body (hatched arrow, Fig. 2a). This segment of the parasite's membrane also appears quite prominent in cells treated by the Con-A horseradish peroxidase-diamonobenzidine technique (CHIARI et al., 1978; MARTINEZ-PALOMO et al., 1976) probably due to its carbohydrate content.

Removal of the linear staining was completed after extraction for 72 hours. Trypsin extraction removed only the globular antigen and the same was found for bloodstream forms of **T. congolense** by JACKSON et al., 1978.

The supernatant from PBS extracted epimastigotes was identical on ID tests with an antigen present in the supernatant from cell cultures infected with **T. cruzi** trypomastigotes (ABRAHAMSOHN & KLOETZEL, in press) and also with one of the bands present in trypsin extracts (Fig. 7). This finding suggests that at least two **T. cruzi** differentiation stages may release their surface protein to either the culture medium or the environment.

From IF results obtained when PBS extracted epimastigotes were used as antigen for such tests and from blocking of IF tests when the supernatant of PBS extracted epimastigotes was mixed to human Chagas'standard sera prior to testing, it seems conclusive that the globular aggregates constitute the antigenic determinants for IF tests. Since extraction procedures removed in lesser time the globular aggregate staining than the linear one (and trypsin extraction removed it exclusively) we can conclude that different antigenic determinants are attached to the cell membrane of **T. cruzi** epimastigotes.

RESUMO

Coloração imunohistoquímica da membrana do Trypanosoma cruzi. **Perda dos determinantes antigênicos após extração salina**

Por métodos imunohistoquímicos empregando soro de coelho anti-**T.cruzi** conjugado à peroxidase e com desenvolvimento de reação por meio de substrato adequado foi possível identificar a presença de dois tipos de determinantes antigênicos na membrana de epimastigotas da cepa do **T. cruzi.** A extração salina por diferentes períodos determinou a remoção dos determinantes. Após extração os parasitas foram fixados a lâminas de microscópio e usados em testes de imunofluorescência onde se verificou que a extração salina determinou remoção dos receptores para tais testes da superfície dos tripanossomas.

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