

Cell-Substrate Adhesion during *Trypanosoma cruzi* Differentiation

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Abstract. The transformation of *Trypanosoma cruzi* epimastigotes to the mammal infective metacyclic trypomastigotes (metacyclogenesis) can be performed in vitro under chemically defined conditions. Under these conditions, differentiating epimastigotes adhere to a surface before their transformation into metacyclic trypomastigotes. Scanning and transmission electron microscopy of adhered and non-adhered parasites during the metacyclogenesis process show that only epimastigotes and few transition forms are found in the first population, whereas metacyclic trypomastigotes are exclusively found in the cell culture supernatant. PAGE analysis of the [³⁵S]methionine metabolic labeling products of adhered and non-adhered parasites shows that although most of the polypeptides are con-

served, adhered parasites express specifically four polypeptides in the range of 45–50 kD with an isoelectric point of 4.8. These proteins might be involved in the adhesion process and are recognized by an antiserum against total adhered parasite proteins. This antiserum also recognized a group of 45–50 kD in the iodine-radiolabeled surface proteins of differentiating cells, providing direct evidence that these components are indeed surface antigens. The results suggest that epimastigotes must adhere to a substrate before their transformation to metacyclic trypomastigotes, being released to the medium as the metacyclogenesis process is accomplished. This could correspond to the process naturally occurring within the triatomine invertebrate host.

THE protozoan parasite *Trypanosoma cruzi* is the etiological agent of American trypanosomiasis or Chagas' Disease (5). The life cycle of this parasite comprises three major stages: epimastigotes, trypomastigotes, and amastigotes. The epimastigotes replicate within the insect host midgut and change to metacyclic trypomastigotes (the process of metacyclogenesis), a nonproliferating form capable of infecting the vertebrate host. Metacyclic trypomastigotes are released in the excreta of the reduviidae insects (triatomines) during the blood meal and can invade the mammalian host through a wound or mucosa and infect different cell types, mainly macrophages and muscle cells, through an endocytic process (2, 8). Inside the cell, metacyclic trypomastigotes differentiate to amastigotes, which are the proliferative form in the mammal host. After several rounds of multiplication, they turn into trypomastigotes and escape from the infected cells. Once in the bloodstream, trypomastigotes may invade new cells or close the cycle upon ingestion by the invertebrate vector, where they transform again into the replicative epimastigotes (2, 8).

The metacyclogenesis process is of special interest since it comprises the morphogenetic transformation of a non-pathogenic to a pathogenic form. This process can be mimicked in vitro using a chemically defined differentiation medium (7). With this medium, biochemical analysis of differentiating cells is feasible and indeed has allowed the identification of stage-specific gene expression products as well as the correlation between gene products and acqui-

sition of trypomastigote stage-specific biological properties. In addition, comparison of triatomine derived with in vitro differentiated metacyclic trypomastigotes shows that both display similar biological properties (7).

The morphogenetic transformation of different higher eukaryotic cells is often preceded by cell-substrate or cell-cell adhesion through cell-adhesion molecules (10). Very little is known about this phenomenon in lower eukaryotes, however a cell adhesion molecule has been described during the differentiation of the slime mold *Dictyostelium discoideum* (12).

In a series of metacyclogenesis experiments, we noticed that *T. cruzi* epimastigotes adhered to the culture flasks before their differentiation to metacyclic trypomastigotes. Interestingly, a similar phenomenon has been observed inside the invertebrate host, where epimastigotes are attached to the epithelium of the insect rectal gland, being released when they transform to metacyclic trypomastigotes (1, 9, 20). In this study, we analyzed the relevance of these observations to the metacyclogenesis process by morphological and biochemical characterization of adhered and non-adhered forms.

Materials and Methods

Parasites

The *Trypanosoma cruzi* Dm 28c clone was obtained and kept in the laboratory as previously described (6).

In Vitro Metacyclogenesis of *T. cruzi*

This was performed under chemically defined conditions as described (7) with the difference that we have used the TAU3AAG medium, which consists of the previously described TAUP (190 mM NaCl; 17 mM KCl; 2 mM MgCl₂; 2 mM CaCl₂; 8 mM phosphate pH 6.0; 10 mM L-proline; 0.035% sodium bicarbonate) supplemented with 50 mM sodium L-glutamate, 2 mM sodium L-aspartate, and 10 mM glucose. Briefly, epimastigotes from LIT medium (3) were harvested by centrifugation at 10,000 g for 15 min at 10°C and resuspended in artificial triatomine urine (TAU; 190 mM NaCl; 17 mM KCl; 2 mM MgCl₂; 2 mM CaCl₂; 8 mM phosphate buffer, pH 6.0; 0.035% sodium bicarbonate) in a concentration of 3–5 × 10⁸ cells/ml. After 2 h of incubation at 28°C, parasites were transferred to culture flasks (25 cm²; Falcon Labware, Oxnard, CA) containing 10 ml of TAU3AAG (final concentration of 3–5 × 10⁶ cells/ml), followed by incubation at 28°C for different times.

Non-adhered Parasites during Metacyclogenesis

The number of non-adhered parasites during the differentiation was determined by counting cells in a hemacytometer.

Scanning Electron Microscopy

For scanning electron microscopy, glutaraldehyde-fixed trypanosomes (2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature) were cross-linked to the surface of glass coverslips previously coated for 15 min with 0.1% poly-L-lysine (Sigma Chemical Co., St. Louis, MO; molecular mass 30,000–70,000 D), then rinsed in PBS (20 mM phosphate buffer, pH 7.5; 150 mM NaCl) and postfixed in 1% OsO₄ for 5 min. Subsequently the cells were dehydrated in ethanol and critical point dried with CO₂. A 20-nm thick layer of gold was deposited on the preparations which were examined in a JEOL 25 S II scanning electron microscope. To observe cells spontaneously adhered to the glass substrate, glass coverslips were introduced into petri dishes used to cultivate the parasites. After different periods of incubation (6, 24, 48, 72, and 96 h), the glass coverslips were removed, washed twice with PBS, fixed in glutaraldehyde (2.5%), and washed again with PBS.

Transmission Electron Microscopy

Non-adhered cells were fixed in 2.5% glutaraldehyde-4% paraformaldehyde solution containing 3% sucrose, in 0.1 M phosphate buffer, pH 7.2 for 1 h at room temperature. Cells adhered to the glass or polystyrene substrate were fixed with glutaraldehyde (2.5%) as above, washed twice with PBS, and removed using a rubber policeman. The cells were postfixed in a solution containing 1% OsO₄, 1 mM CaCl₂, 0.8% potassium ferricyanide, and 3% sucrose in 0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature. Thereafter, the cells were washed in buffer, incubated for 1 h in an aqueous solution containing 2% uranyl acetate, dehydrated in ethanol, and embedded in Epon. Thin sections were stained for 20 min with uranyl acetate and 5 min with lead citrate and observed with a JEOL 100 C-X electron microscope.

Cell Electrophoresis

The electrophoretic mobility of the cells was determined in a Zeiss Cytopherometer with a current of 4–6 mA and a final voltage of 100 V. The cell suspension (previously fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer) was placed into the chamber and then allowed to equilibrate for 10 min. Measurements were made at a temperature of 25°C in a 0.85% NaCl solution (ionic strength 0.145 mol.dm⁻³) at pH 7.2. When current was switched on, we measured the time necessary for one cell to travel across two vertical lines which correspond to a distance of 16 μm. Then the polarity was reversed, and the same cell was measured again traveling in the opposite direction. 50–100 cells were measured in each sample analyzed. Calibration of the equipment was made by measuring the electrophoretic mobility of fresh human erythrocytes.

1. *Abbreviations used in this paper:* LIT, liver infusion tryptose; TAU, artificial triatomine urine (190 mM NaCl, 17 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 8 mM phosphate buffer pH 6.0, 0.035% sodium bicarbonate).

In Vivo Labeling of Parasites with [³⁵S]Methionine

We have used two alternative protocols for the metabolic labeling of parasites with [³⁵S]methionine. In the first, the epimastigotes were labeled before incubation in the differentiating medium and then allowed to differentiate in TAU3AAG. This method will be referred to as "prelabeling" of parasites comprising both adhered and non-adhered cells. In the second, parasites from different times of differentiation in TAU3AAG were divided into two populations (adhered and non-adhered) and then separately submitted to metabolic labeling with [³⁵S]methionine. The latter will be referred to as "pulse labeling" of parasites.

Prelabeling Method. 3 × 10⁸ parasites from a final log phase culture in LIT medium (3) were centrifuged at 10,000 g for 15 min at 10°C. The cells were incubated in 1 ml of artificial triatomine urine (TAU) for 1 h at 28°C. Cells were harvested as above and then incubated in 1 ml of TAU3AAG medium supplemented with 100 μCi [³⁵S]methionine (1,045 Ci mol⁻¹; Amersham International, Amersham, UK) for 2 h at 28°C. 2.5 × 10⁷ parasites (100% of epimastigote forms) were then incubated in 5 ml of TAU3AAG medium in culture flasks (25-cm² surface area) and thereafter at 28°C for different times (6, 12, 18, 24, 48, 72, and 96 h). At each time point, the medium containing non-adhered parasites was removed and centrifuged at 10,000 g for 15 min at 4°C. Non-adhered pelleted cells were resuspended in 0.5 ml of lysis solution (150 mM NaCl; 10 mM Tris HCl, pH 7.5; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride [PMSF]; 2 mM iodoacetamide; 1% NP-40) and added back to the culture flask containing adhered cells in 1.5 ml of lysis buffer. The labeled parasites were vigorously shaken for 3 min at room temperature and centrifuged for 10 min at 12,000 g. The supernatant was immediately processed for immunoprecipitation or electrophoresis or alternatively stored at -20°C until use. We have also included in our analysis parasites (2 × 10⁷ cells) labeled in TAU medium (0 h of differentiation) or in TAU3AAG medium (2 h of differentiation) as above.

Pulse Labeling Method. Parasites remaining in the supernatant from different times of incubation in TAU3AAG medium (24, 48, 72, and 96 h) were harvested by centrifugation at 10,000 g for 15 min at 4°C. The cells (2 × 10⁷) were incubated for 2 h at 28°C in 1 ml of TAU3AAG medium supplemented with [³⁵S]methionine. The adhered cells from each time of differentiation were washed with TAU3AAG medium and incubated for 2 h at 28°C in 2 ml of TAU3AAG medium supplemented with [³⁵S]methionine. After the incubation, the different samples were separately processed. Non-adhered cells were washed twice with 2 ml of TAU3AAG medium, centrifuged at 12,000 g for 5 min, and lysed in 0.5 ml of lysis solution as described above. Adhered cells were washed twice with 5 ml of TAU3AAG and then lysed by the addition of 2 ml of lysis solution and vigorous shaking for 3 min at room temperature. The samples were processed for electrophoresis and immunoprecipitated as described below.

Surface Iodination of the Cells

Surface proteins of the parasites were labeled with ¹²⁵I by the iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Pierce Chemical Co., Rockford, IL) procedure (4, 11). Briefly, 10⁸ cells were washed three times in cold PBS and resuspended in a final volume of 150 μl of PBS. The suspension was transferred to an iodogen-coated (0.5 mg) glass test tube. 200 μCi of carrier-free ¹²⁵I (2,000 Ci.mmol⁻¹; Amersham International) were then added and the sample incubated in an ice bath for 10 min with constant agitation. The sample was then removed and washed again three times with cold PBS. The cells were then lysed in 0.5 ml of lysis solution as described above.

Preparation of Antisera and Immunoprecipitation

Trypomastigote stage-specific antiserum was prepared as previously described (7). Antiserum directed against adhered parasites was prepared in New Zealand rabbits as follows. After 6 h of parasites differentiation in TAU3AAG, the non-adhered cells were removed, and the cells that adhered to the glass were washed twice with TAU3AAG and left 15 min at 4°C. They were then removed using a rubber policeman. 10⁸ adhered cells were resuspended in 2 ml of Freund's complete adjuvant and injected in rabbits, in intervals of 8 d, first subcutaneously in the back, and then intramuscularly. After a week, the rabbits were inoculated intravenously three times at 48 h intervals with 10⁷ adhered parasites in TAU3AAG medium. 6 d after the last inoculation, the animals were bled and the antiserum was obtained. Immunoprecipitations were carried out as previously described (7).

PAGE and Fluorography

The proteins were analyzed by one-dimensional electrophoresis on linear gradients of 10–15% polyacrylamide gels containing SDS (14). Alternatively, the samples were analyzed by two-dimensional PAGE (2D-PAGE) (16). Gels were treated for fluorography (15) and autoradiographed at -70°C using a Kodak X-Omat intensifying screen.

Results

T. cruzi metacyclic trypomastigotes can be easily and reproducibly obtained after the incubation of epimastigotes in the chemically defined TAUP (7) or TAU3AAG (13) media. However, low yields of metacyclic trypomastigotes were obtained when the axenic culture metacyclogenesis was performed either under constant agitation or in siliconized flasks (results not shown), suggesting that the adhesion of epimastigotes to culture flasks might be an important step in enabling the parasites to transform into metacyclic trypomastigotes.

Since epimastigotes adhere to triatomine intestine membranes before their transformation into metacyclic trypomastigotes (20), we have studied, under *in vitro* chemically defined conditions, the adhesion of the epimastigotes to the culture flask before their transformation to metacyclic trypomastigotes, and characterized the differentiating cells in terms of adhesion kinetics, morphology, surface charge, and gene expression products.

Quantification of the Parasites during the *In Vitro* Metacyclogenesis

As a first step to evaluate the relevance of the epimastigote adhesion to the metacyclogenesis process, we determined, by differential counting, the number of parasites that remained in suspension in TAU3AAG after the transfer of LIT medium epimastigotes to the chemically defined differentiating solution, at different times of the *in vitro* metacyclogenesis process. The results showed a drastic reduction of the number of parasites in the supernatant after 6 h of differentiation (Fig. 1). This reduction was due to the adhesion of the epimastigotes to the culture flask as visualized by light microscopy. With prolonged times of incubation, there was an increasing number of parasites in the supernatant. Differential counting of the parasites showed that metacyclic trypomastigotes were observed from 24 h on. The metacyclogenesis process was essentially complete at 96 h since after longer times of incubation, the total number of metacyclic trypomastigotes did not increase. These results suggested that the differentiating cells were released from the support to the medium as metacyclic trypomastigotes.

Scanning Electron Microscopy

With the aim to look at the morphology of adhered and free parasites and the overall aspects of adherence, we examined the differentiating cells by scanning electron microscopy. The parasites derived from LIT medium had a morphology typical of epimastigotes (Fig. 2). However, after 6 h of incubation in TAU3AAG, most of the adhered parasites were observed in clusters as epimastigotes in the process of division showing two flagella (Figs. 2–4). Parasites spread over the substrate preferentially through flagellar extensions (Fig. 4). Longer incubation times (24, 48, 72, and 96 h) did not significantly change the general morphology of most of the

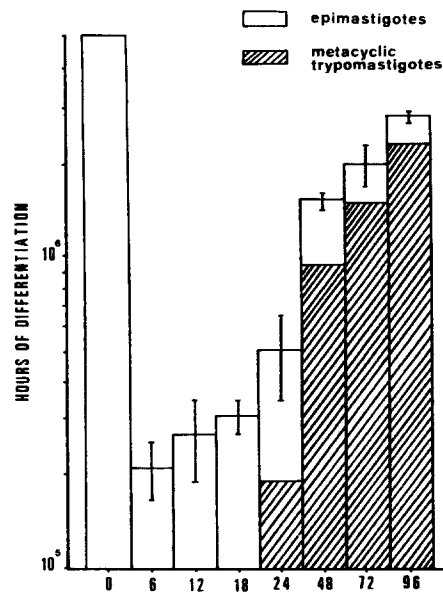


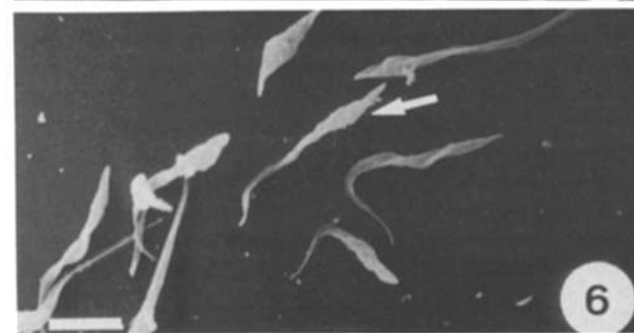
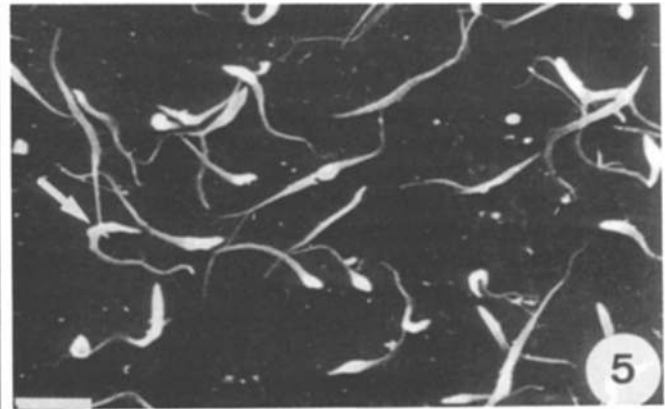
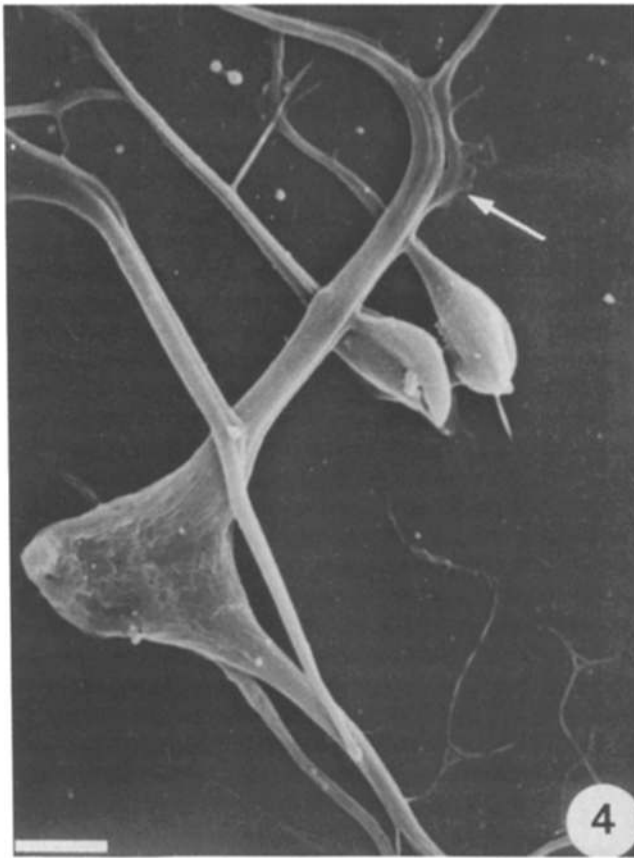
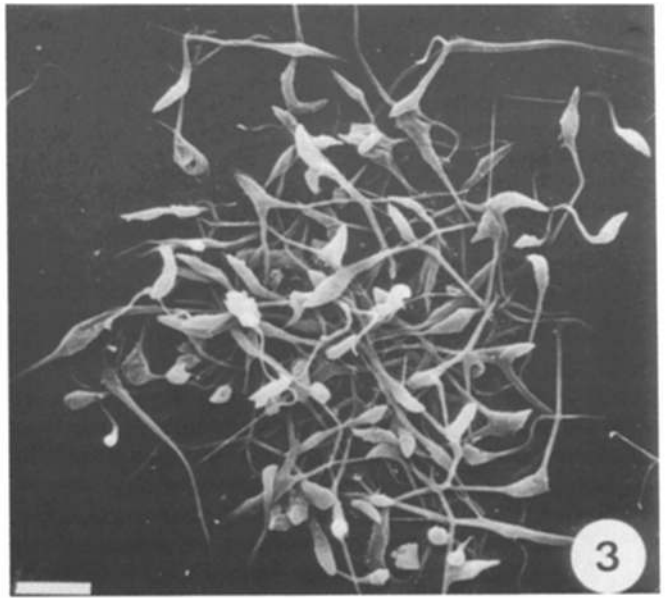
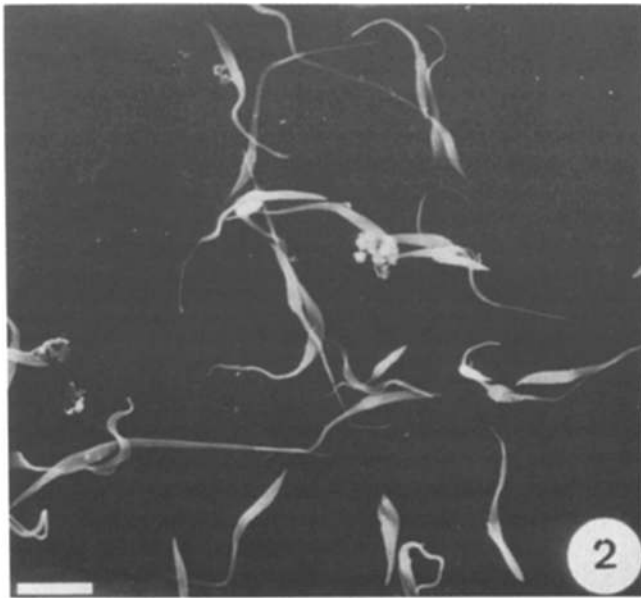
Figure 1. Quantification of non-adhered cells during the *in vitro* metacyclogenesis of *T. cruzi*. The number of non-adhered parasites was determined by differential cell counting in a Neubauer chamber. The inoculum was estimated to be $5 \times 10^6/\text{ml}$. Mean and standard error are shown. $n = 6$.

adhered parasites, although some became thinner than epimastigotes with a morphology that might correspond to intermediate forms between epimastigotes and trypomastigotes (Fig. 6).

The analysis of the free parasites in the supernatant showed that their general morphology changed with time. They became thinner, and a large number of intermediate and trypomastigote forms were observed (Fig. 7). With prolonged times of incubation, the percentage of metacyclic trypomastigotes became increasingly greater (46, 77, and 92% for 48, 72, and 96 h, respectively), corroborating the above-mentioned suggestion that parasites are released to the culture medium as they transform to trypomastigotes.

Transmission Electron Microscopy

To support the scanning electron microscopy data, it was important to determine whether adhered cells displayed an epimastigote ultrastructure and whether thinner parasites corresponded to intermediate forms. This was performed by transmission electron microscopy, and it was observed that in most of the parasites attached to the substrate, the kinetoplast had a rodlike shape, characteristic of epimastigotes (Figs. 8 and 9). However, we could also observe a rod-shaped kinetoplast laterally located in relation to the nucleus, characteristic of intermediate forms (Fig. 12). On the other hand, most of the parasites found in the supernatant after 96 h of incubation displayed a round kinetoplast characteristic of trypomastigote form. These forms were already observed after 24 h of incubation (Figs. 10 and 11) and their number increased with time. Intermediate forms were also observed (Fig. 12), and in some cases a close contact between the nucleus and the kinetoplast was observed (Fig. 13).



Electrophoretic Mobility

Epimastigotes and trypomastigotes possess distinct electrophoretic mobilities (17). To further characterize the forms observed during the differentiation and check the relevance of surface charges to the adhesion process, we have analyzed the electrophoretic mobility of differentiating cells. Our observations indicated that under standard conditions (ionic strength of $0.145 \text{ mol} \cdot \text{dm}^{-3}$, pH 7.2, 25°C) cells had a negative charge with the magnitude depending on the morphology of the parasites obtained at different times of incubation in TAU3AAG medium (Fig. 14). As previously observed for other strains of *T. cruzi* (17), the orientation of Dm 28c non-adhered cells was random during migration towards the positive electrode; some moved with the anterior end towards the cathode, others with the posterior end, and others laterally. Populational analysis indicated that after 48, 72, and 96 h of differentiation, ~ 46 , 77, and 92%, respectively, of the parasites present in the medium displayed electrophoretic mobilities typical of those found earlier for trypomastigotes (17). These changes in the electrophoretic mobility might reflect changes occurring at the level of cell surface proteins, possibly relevant to the adhesion process.

Protein Synthesis during the Metacyclogenesis Process

Analysis of polypeptides synthesized by differentiating cells might allow the identification of those transiently expressed and likely to be involved in the adhesion process. The comparison by 2D-PAGE of the protein synthesis pattern of parasites during the metacyclogenesis in TAU3AAG showed that the most striking difference between the patterns was that a group of polypeptides displaying an isoelectric point of 4.8 and a molecular mass ranging from 45–50 kD appeared after 6 h of differentiation (13). In addition, the expression of these polypeptides decreased with the differentiation time.

Additional evidence that these proteins of the 45–50 kD group might be involved in the adhesion process was obtained by immunoprecipitating the metabolically labeled products from differentiating cells with an antiserum raised against 6 h adhered parasites (Fig. 15). The results showed that a 45–50-kD polypeptide was immunoprecipitated from differentiating cell extracts of 6, 24, and 48 h (Fig. 15 B, lanes 3–5, respectively). This polypeptide was barely detectable at 72 and 96 h (Fig. 15 B, lanes 6 and 7, respectively) and was absent at 0 and 2 h (Fig. 15 B, lanes 1 and 2, respectively). Interestingly, the antiserum recognized also a 60-kD polypeptide in 0 h differentiating cells (Fig. 15 B, lane 1). A longer exposure of the same gel (Fig. 15 A) showed a complex pattern as expected for an immunoprecipitation with an antiserum obtained against total parasites.

The results described above indicated that some polypeptides were transiently expressed during the metacyclogenesis process. To investigate whether this transition polypep-

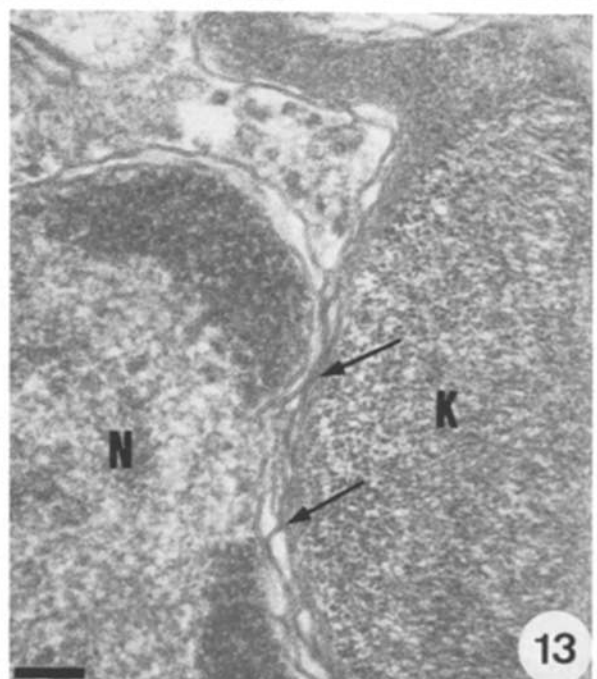
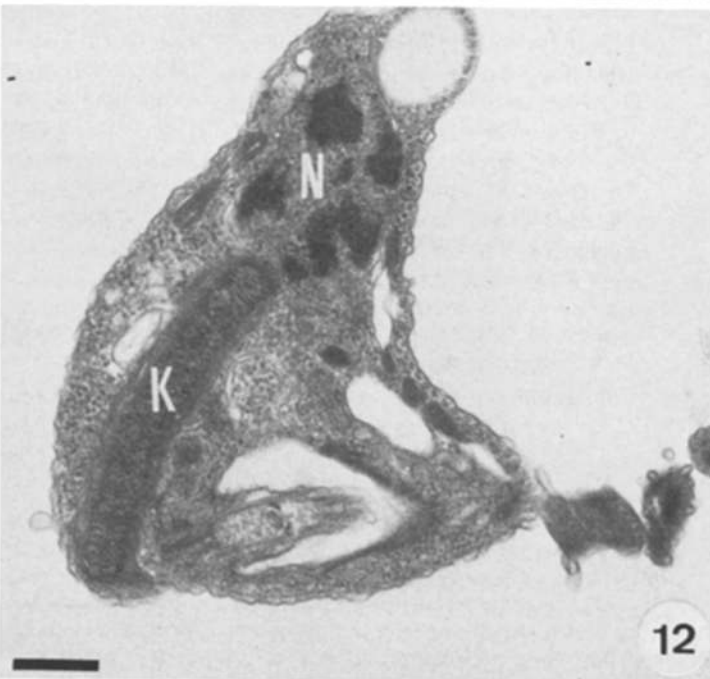
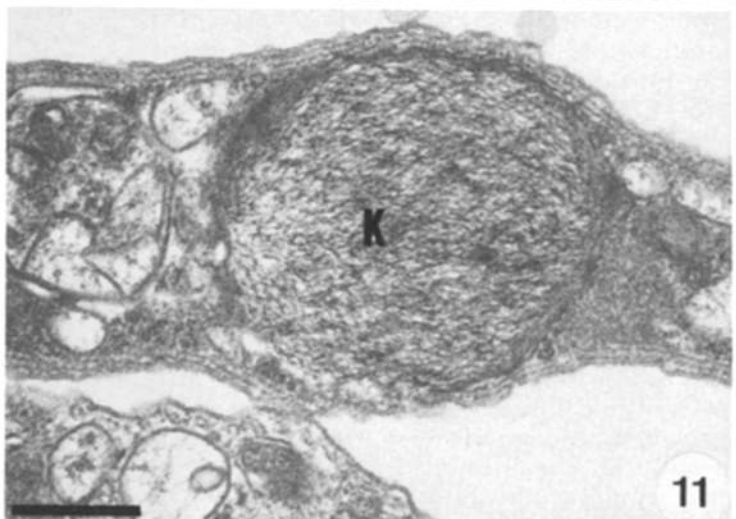
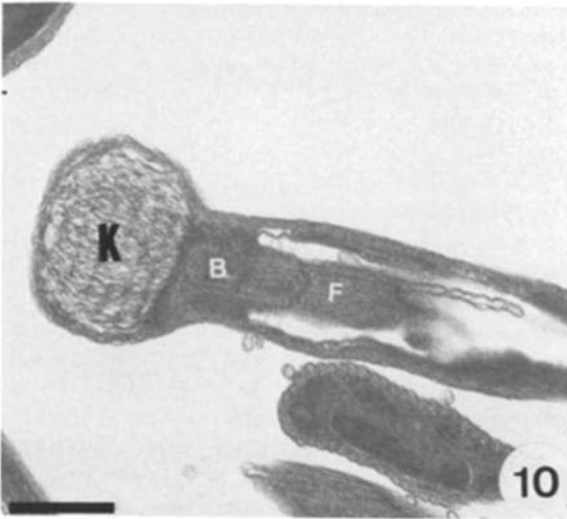
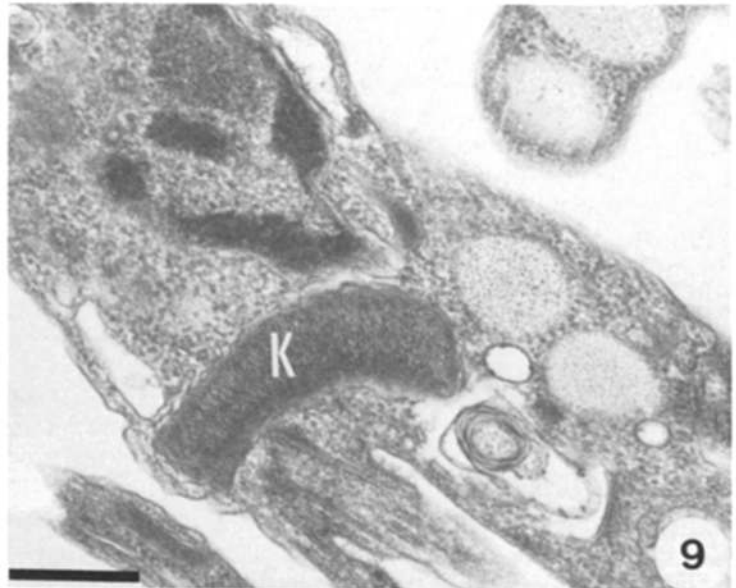
tides might result from genes specifically expressed in adhered cells, we have analyzed, by 2D-PAGE, metabolically labeled products derived from adhered and non-adhered cells after 24 and 96 h of incubation in TAU3AAG medium. Parasites from each two points were isolated and labeled with [^{35}S]methionine (see Pulse Labeling Method in Materials and Methods).

Adhered 24 h differentiating cells displayed a complex protein pattern (Fig. 16, *top left*). The comparison of this pattern with the others showed the presence of two groups of polypeptides barely detectable in 24 h non-adhered and 96 h adhered cells (Fig. 16, *bottom left* and *top right*, respectively) and absent in 96 h non-adhered cells (Fig. 16, *bottom right*). Group 1 was composed of four acidic polypeptides, two of 45 kD and two of 50 kD, with an isoelectric point of 4.8, while group 2 was composed of two polypeptides with respective molecular masses of 54 and 57 kD and an isoelectric point of 5.3. This result suggests that these proteins might be involved in the adhesion of the epimastigotes, which is in itself a step required for differentiation to metacyclic trypomastigotes.

The other main difference in the two-dimensional protein pattern was the presence of polypeptides with molecular masses in the range of 75–90 kD (Fig. 16, *bottom right*). These polypeptides are characteristic of metacyclic trypomastigotes (7), in agreement with the morphological and ultrastructural data showing that this population is highly enriched in trypomastigotes.

As shown above, most of the adhered cells displayed a typical epimastigote morphology and ultrastructure. In addition, the metabolic labeling products from adhered cells (Fig. 16, *top left* and *top right*) differed from the pattern of 96-h non-adhered cells (Fig. 16, *bottom right*), the latter having a polypeptide pattern characteristic of metacyclic trypomastigotes. However, previous results from our laboratory (6) have shown that cells morphologically characterized as epimastigotes may, under certain conditions, express trypomastigote stage-specific gene products. Therefore, we have further characterized the adhered parasites in terms of surface antigens expression. For this purpose, metabolically labeled products from adhered and non-adhered cells were immunoprecipitated with a trypomastigote stage-specific antiserum, which specifically recognizes two surface antigens of 78 and 86 kD (7). The results shown in Fig. 17 include different times of metacyclic trypomastigote induction (24, 48, 72, and 96 h). Only non-adhered parasites (lanes 1, 3, 5, and 7, respectively) express trypomastigote stage-specific surface antigens. This result corroborates our previous finding that adhered parasites consist mainly of epimastigotes and intermediate forms, which are probably released in the supernatant as they transform to metacyclic trypomastigotes.

Figures 2–7. Scanning electron micrographs of parasites that remained attached to glass coverslips introduced into the culture flask (Figs. 3–6) or were found in the supernatant (Fig. 7). The control parasites derived from LIT medium showed a typical epimastigote form (Fig. 2). Dividing forms were observed in parasites attached to the glass coverslips after an incubation of 6 h (Figs. 3–4). Filopodium-like surface projections (*arrows*) were seen in some of these parasites (Fig. 4). Intermediate forms between epimastigotes and trypomastigotes were observed as attached parasites (*arrows*) after an incubation time of 48 (Fig. 5) or 96 h (Fig. 6). Most of the parasites found in the supernatant of cultures maintained for 96 h (Fig. 7) had a typical trypomastigote form. The region of the kinetoplast (*K*), which is located in the posterior portion of the protozoan, could be easily identified. Bars: (Figs. 2, 3, 5, and 6) 10 μm ; (Figs. 4 and 7) 2 μm .



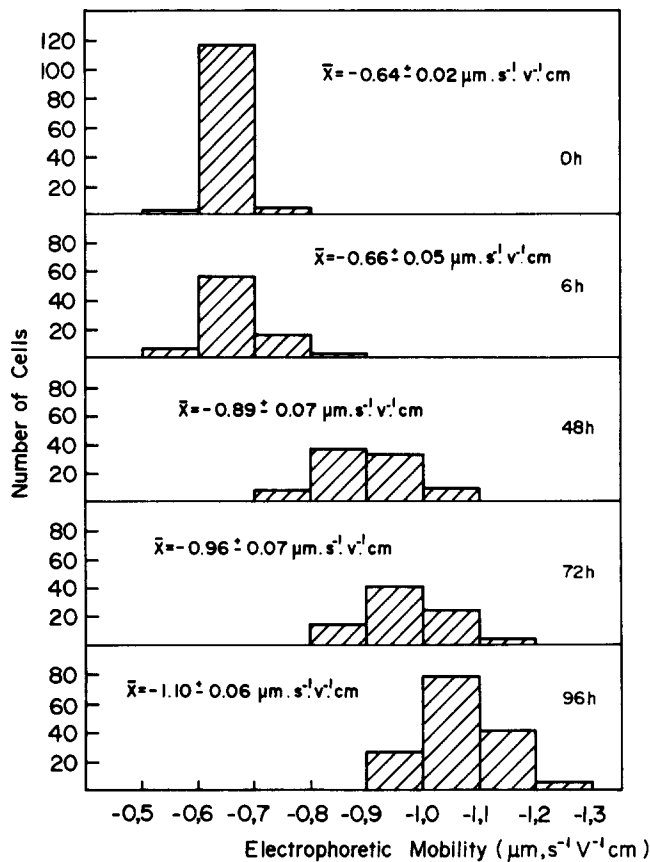


Figure 14. Distribution of the electrophoretic mobility of parasites found in the supernatant of cultures incubated for different periods. For cell measurement, $I = 0.145 \text{ mol. dm}^{-3}$, pH 7.2.

Pattern of Surface Proteins during the Differentiation

To identify the changes occurring in the surface of the parasites during the metacyclogenesis process, and to analyze whether the putative adhesion proteins of 45- to 50-kD group are surface proteins, we have labeled differentiating cells with ^{125}I . PAGE of total iodinated proteins during different times of the metacyclogenesis process showed a complex pattern and did not allow the detection of any change in the range from 45 to 50 kD (results not shown). However, immunoprecipitation of iodine-labeled 24-h adhered cells with the 6-h adhered parasites antiserum allowed the detection of a group of polypeptides in the range from 45 to 50 kD (Fig. 18, lane 2), which was barely detected in LIT medium epimastigotes (Fig. 18, lane 1) and was absent from iodine-labeled metacyclic trypomastigotes (Fig. 18, lane 3). Indeed, 2D-PAGE of the polypeptides immunoprecipitated with the adhered-cells antiserum from iodine-labeled 24-h differentiating cells showed the identity between this pattern (Fig.

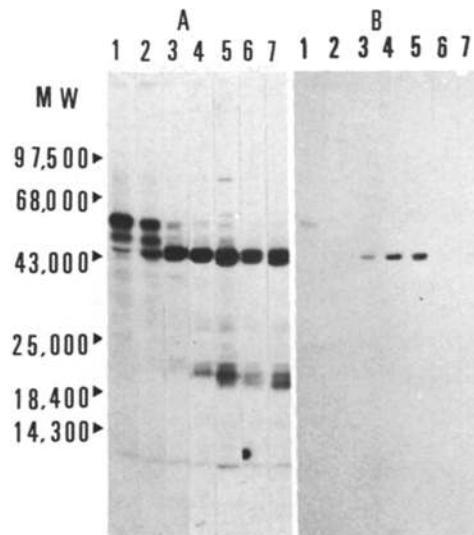


Figure 15. 10–15% linear gradient SDS-PAGE of the polypeptides (pre-labeling method) immunoprecipitated with the antiserum against adhered parasites during in vitro metacyclogenesis of *T. cruzi*. A and B correspond to two different times of exposure of the same gel. Lanes 1–7 refer to total cells from 0, 2, 6, 24, 48, 72, and 96 h of differentiation in TAU3AAG medium, respectively. The immunoprecipitations were carried out using 10^6 cpm. The position of ^{14}C molecular weight markers is shown on the left side of the figure.

19) and that obtained for ^{35}S methionine-labeled differentiating cells (Fig. 16).

Discussion

Studies carried out in the invertebrate host showed that epimastigotes are found adhered to the surface of the intestine epithelial cells (1, 9, 20). Our data show, for the first time, that under in vitro differentiating conditions, epimastigotes of *T. cruzi* must adhere to a substrate before their transformation to metacyclic trypomastigotes. These observations were made under the chemically defined in vitro differentiating conditions previously described (7). The adhesion occurs with different strains and clones of *T. cruzi*. This adhesion seems to be necessary, but not sufficient, for the metacyclogenesis process, since we have observed a clone that adhered but did not transform to metacyclic trypomastigote (unpublished results). Low yields of metacyclic trypomastigotes were obtained when the metacyclogenesis was carried out in siliconized flasks or with constant stirring (results not shown), reinforcing the importance of the cell-substrate adhesion during *T. cruzi* differentiation. Therefore, the condition we have established, using a chemically de-

Figures 8–13. (Figs. 8 and 9) Thin sections of parasites that remained attached to the glass coverslips after an incubation time of 24 or 96 h, respectively. The kinetoplast DNA network (*K*) has a rodlike aspect characteristic of epimastigotes. Bars, 0.5 μm . (Figs. 10 and 11) Parasites found in the supernatant of cultures incubated for 24 h had a round kinetoplast (*K*) characteristic of trypomastigotes. *B*, basal body; *F*, flagellum. Bars, 0.5 μm . (Figs. 12 and 13) Many intermediate forms between epimastigotes and trypomastigotes were seen in the supernatant of cultures incubated for periods varying from 24 to 48 h. The kinetoplast (*K*) is laterally located in relation to the nucleus (*N*). Close contact between these two structures was observed (arrows in Fig. 13). Bars: (Fig. 12) 0.5 μm ; (Fig. 13) 0.1 μm .

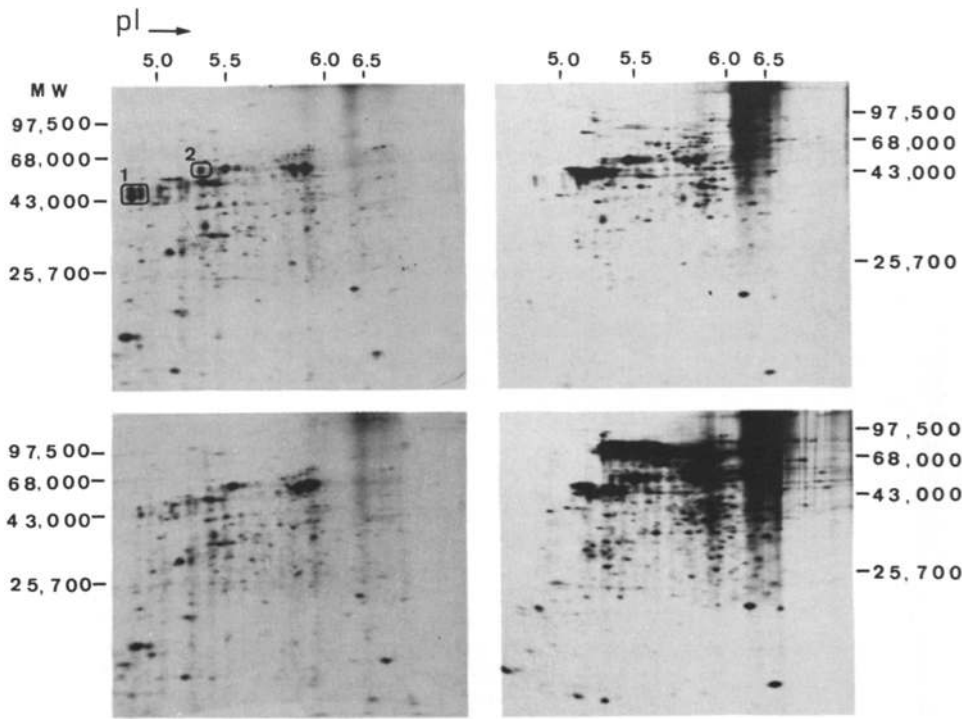


Figure 16. Two-dimensional PAGE of the [³⁵S]methionine pulse labeled products (see Materials and Methods) from *T. cruzi* Dm 28c cells in TAU3AAG medium. (Top left) Adhered cells of 24 h. (Top right) Adhered cells of 96 h. (Bottom left) Non-adhered cells of 24 h. (Bottom right) Non-adhered cells of 96 h. First dimension is an isoelectric focusing and second dimension is a uniform 13% SDS-PAGE. Each gel was loaded with 10⁶ cpm. The numbers at the horizontal axis refer to the pH gradient and at the vertical axis to the ¹⁴C molecular weight markers.

fined medium, provides a simple biological system that seems to reproduce in vitro a complex biological phenomenon fundamental for *T. cruzi* to exert its pathogenic effect in the vertebrate hosts.

The scanning electron micrographs show that attached cells tend to spread onto the substrate, with the formation of some projections. These were more evident in the flagellum, as shown in Fig. 4. Previous studies have shown that it is mainly through the flagellum that trypanosomatids attach to biological substrates and that expansions of the flagellar membrane are observed in the attachment region (19).

Both electron microscopy observations and cell electrophoresis experiments indicate that the transformation of epimastigotes to trypomastigotes might mimic the process naturally occurring inside the invertebrate host. The observed changes in the position of the kinetoplast relative to

the nucleus and the structural organization of the kinetoplast DNA network are similar to that observed in more complex biological systems (8). In addition, during the differentiation process, the surface of the parasite gradually becomes more negative. Trypomastigotes obtained after 96 h of incubation have a mean cellular electrophoretic mobility similar to that of trypomastigotes obtained from the bloodstream of mice infected with *T. cruzi* or from the supernatant of vertebrate cells infected in vitro with *T. cruzi* (17, 18). However, we do not believe that the adhesion of the parasites is due to the net charge of the culture flasks since epimastigotes adhere irrespective to the coating of the surfaces with poly-L-lysine or poly-L-glutamic acid (data not shown).

After 96 h, a large number of transition forms, which can be easily recognized in thin sections, were seen among the attached parasites. Observations made with the scanning electron microscope in the posterior intestine of *Rhodnius prolixus* infected with *T. cruzi* did not show trypomastigotes attached to the epithelial surface in contrast to what occurs with epimastigotes (1, 20). Taken together these observations

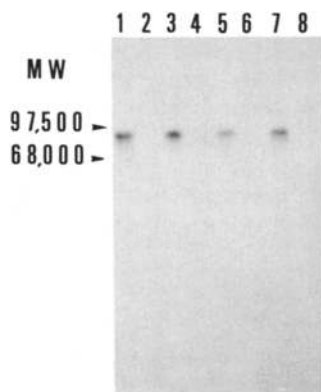


Figure 17. 10-15% linear SDS-PAGE of the polypeptides (pulse labeled method, see Materials and Methods) immunoprecipitated with trypomastigote stage-specific antiserum from *T. cruzi* Dm 28c differentiating in TAU3AAG medium. Lanes 1, 3, 5, and 7 refer to non-adhered parasites of 24, 48, 72, and 96 h of incubation, respectively. Lanes 2, 4, 6, and 8 refer to adhered parasites of 24, 48, 72, and 96 h, respectively. Immunoprecipitations were carried out using equal amounts of cpm incorporated into labeled proteins. The positions of ¹⁴C molecular weight markers are shown on the left of the figure.

precipitations were carried out using equal amounts of cpm incorporated into labeled proteins. The positions of ¹⁴C molecular weight markers are shown on the left of the figure.

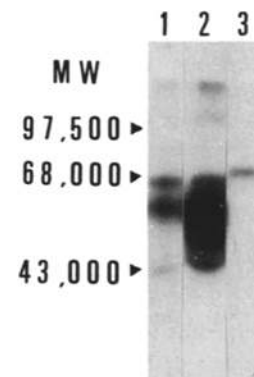


Figure 18. 7-15% linear gradient SDS-PAGE of total surface polypeptides labeled with ¹²⁵I and immunoprecipitated with the antiserum against adhered parasites. Lanes: 1, epimastigotes from LIT medium; 2, adhered cells of 24 h; 3, non-adhered cells of 96 h (metacyclic trypomastigotes). The immunoprecipitations were carried out using 10⁵ cpm. The position of ¹⁴C molecular weight markers is shown on the left side of the figure.

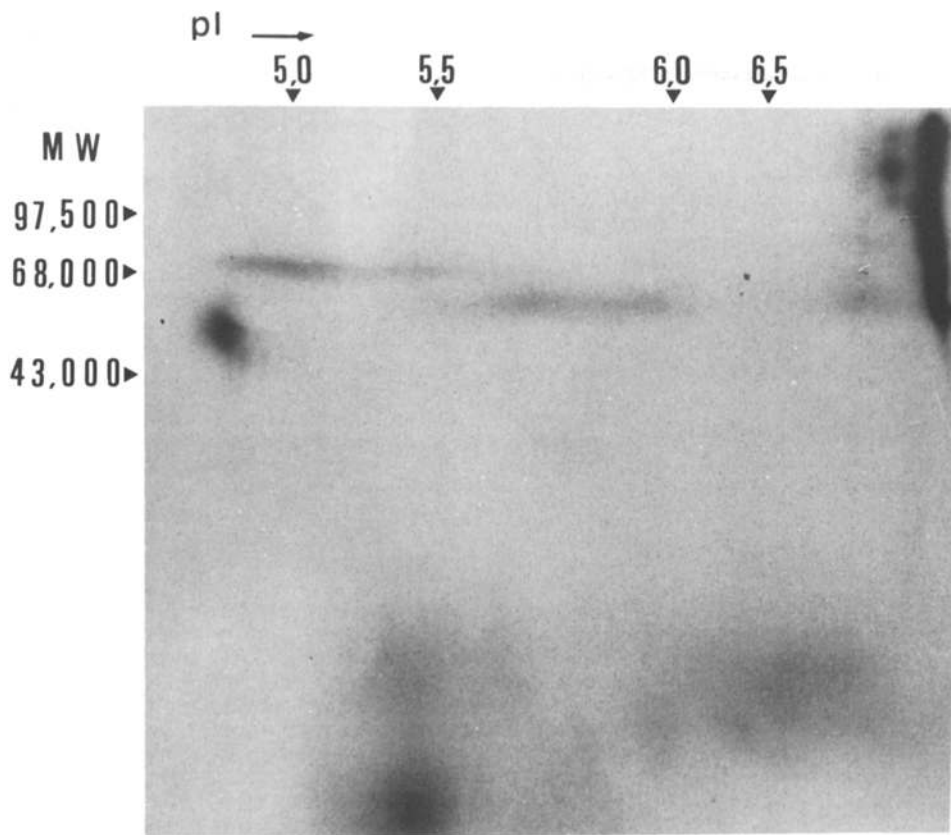


Figure 19. 2D-PAGE of the ^{125}I -labeled surface polypeptides of 24-h adhered parasites immunoprecipitated with the antiserum against adhered cells. First dimension is an isoelectric focusing and second dimension is an uniform 10% SDS-PAGE. The immunoprecipitation was carried out using 10^6 cpm. The numbers at the horizontal axis refer to the pH gradient and at the vertical axis to the ^{14}C molecular weight markers.

suggest that during the epimastigote-trypomastigote transformation, changes take place that lead to the disappearance or blockage of components involved in the attachment of the parasites to substrates.

These components are very likely polypeptides, as suggested by the comparison of the metabolic labeling products from adhered and non-adhered cells during *T. cruzi* metacyclogenesis (Fig. 16). Indeed, the results show that adhered parasites are characterized by the expression of a group of four polypeptides with molecular masses ranging from 45 to 50 kD (isoelectric point of 4.8) and by the expression of a group consisting of two polypeptides with respective molecular masses of 54 and 57 kD and an isoelectric point of 5.3 (Fig. 16). The low level of expression of these polypeptides in non-adhered 24-h differentiating cells (Fig. 16 bottom left) is probably due to the fact that weakly adhered parasites have been released while harvesting the cells. Alternatively, it could be due to the nonsynchronicity of trypanosomes entering the differentiation process.

The immunoprecipitation of proteins of differentiating cells with the adhered parasites antiserum (Fig. 15) provides further evidence on the direct relationship between the 45-50-kD group and the adhesion of the parasites during the metacyclogenesis process. Indeed, these polypeptides are immunoprecipitated from cell extracts of 6-48 h of differentiation and are barely detected at later times and not seen earlier than 2 h. The presence of a 60-kD polypeptide detected in 0-2 h cells, as observed by immunoprecipitation with the adhesion antiserum, deserves further investigation since this 60-kD polypeptide might be a precursor of the 45-50-kD putative adhesion proteins. Accordingly, we have observed

that the 45-50-kD polypeptides became stronger as the 60-kD polypeptide became fainter in [^{35}S]methionine prelabeled parasites (results not shown).

The 45-50-kD putative adhesion proteins are very likely surface antigens in view of both their immunoprecipitation from iodine-labeled adhered cells with the adhered cells antiserum (Fig. 18, lane 2) and their pattern on 2D-PAGE (Fig. 19).

We are presently investigating whether these putative adhesion proteins are glycosylated, since the adhesion of the parasites to the triatomine intestine membranes (20) might be mediated by lectins. However, since the parasites adhered strongly to glass or plastic culture flask, we believe this is not likely to be the case in the *in vitro* differentiating conditions described by us.

Cell adhesion molecules have been described in other eukaryotic cells (10, 12). These molecules play an important role in morphogenetic transformations of differentiating cells. The putative *T. cruzi* cell adhesion molecules differ from those of other eukaryotes on the basis of their respective molecular masses (10, 12). As yet, cell adhesion molecules from other parasites have not been characterized, rendering speculative the idea that they are ancestors of other eukaryotic cell adhesion molecules.

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