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MAJOR SURFACE PROTEINS AND ANTIGENS ON THE DIFFERENT IN VIVO AND IN VITRO FORMS OF *TRYPANOSOMA CRUZI*

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The surface proteins of six stages of *Trypanosoma cruzi* were labeled by Iodogen-catalized surface iodination and analyzed by one and two dimensional polyacrylamide gel electrophoresis. These stages included bloodstream trypomastigotes, culture-form trypomastigotes, amastigotes, staphylomastigotes, epimastigotes, and metacyclic trypomastigotes. Antigens recognized by serum antibodies were detected by Western blotting against serum from mice hyperimmunized against bloodstream trypomastigotes. Bloodstream trypomastigotes, culture-form trypomastigotes and staphylomastigotes contained several surface proteins of molecular weight (M_r) 90 000 and isoelectric points (p*I*) between 5.0 and 7.5. Western blotting reveals that at least two proteins of 90 000 M_r represent the major antigens seen on bloodstream trypomastigotes, culture-form trypomastigotes, staphylomastigotes and amastigotes. However, a 90 000 M_r protein was not detected by either Western blotting or surface iodination on epimastigotes or metacyclic trypomastigotes. The major surface proteins on these latter two stages were represented by several 72 000 M_r proteins with p*I* values between 5.2 and 5.8. An interesting result of this survey is that a 90 000 M_r surface antigen was present on staphylomastigotes, a stage which can be grown in cell free medium.

Key words: *Trypanosoma cruzi*; Cell surface proteins

INTRODUCTION

Trypanosoma cruzi is characterized by a complex life cycle expressing multiple forms of development in both the vertebrate host and the invertebrate vector [1]. The cell surface proteins of several of these different forms have been analyzed with regard to their relative molecular weights (M_r) and recognition by serum antibodies from infected hosts [2–6]. These studies indicate that both qualitative and quantitative

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Abbreviations: kDa, kilodaltons; M_r, molecular weight; p*I*, isoelectric point; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethyl; PBS, 0.02 M sodium phosphate, pH 7.4, 0.9% sodium chloride; DNAse, pancreatic deoxyribonuclease I; RNAse, pancreatic ribonuclease A.

differences exist among the cell surface proteins of the various stages of the life cycle of *T. cruzi*. Although there is general agreement that the major antigenic species on the surface of the bloodstream trypomastigote is an approx. 90 000 M_r glycoprotein, it is unclear whether this protein is present on all developmental stages of the parasite.

In view of this uncertainty as well as to provide necessary information for future studies on the regulation of expression of gene sequences which encode *T. cruzi* surface antigens, we chose to investigate the surface components of six observed morphological forms of *T. cruzi*. In this article, we define by the criteria of relative molecular weight, isoelectric point and recognition by serum antibodies from *T. cruzi* infected mice, the proteins present on the surface of the following in vivo or in vitro forms of *T. cruzi*: (1) bloodstream trypomastigotes isolated from infected mice; (2) culture-form trypomastigotes grown in vitro in vertebrate tissue culture cells; (3) amastigotes isolated from in vitro cultured vertebrate tissue culture cells; (4) staphylomastigotes grown in vitro under acellular culture conditions, (5) epimastigotes grown in monophasic culture and (6) metacyclic trypomastigotes obtained by transformation of epimastigotes in an acellular medium.

MATERIALS AND METHODS

Solutions. Membrane dissolving buffer: 1% Triton X-100, 20 mM Tris-base, pH 7.6, 25 mM NaCl, 50 mM $MgCl_2$, 2 mM phenylmethylsulfonylfluoride, 15 mM 2-mercaptoethanol. 2 × SDS sample buffer: 5% SDS, 8 M urea, 50 mM Tris-HCl pH 6.7, 5% 2-mercaptoethanol, 5 mM EDTA, 10% glycerol, 0.01% bromophenol blue [8]. Phosphate-buffered saline glucose (PSG) solutions were made according to Lanham and Godfrey [9].

Parasites. T. cruzi, Peru strain [10] were used. The parasite developmental form was determined by phase contrast microscopy of live cells and by staining as described [10]. All culture forms were isolated to >98% purity as determined by the above criteria.

Growth and isolation of developmental forms. Mice, C57BL/6, were inoculated with 10^4 bloodstream trypomastigotes and bled about 12 days later by heart puncture using trisodium citrate saline as an anticoagulant [11]. The bloodstream trypomastigotes were separated from red blood cells by a Percoll step gradient [12], rinsed twice in trisodium citrate saline and separated from host cells and platelets by passage over a 5 ml DEAE-cellulose column equilibrated with PSG ($I = 0.206$.)

Culture-form trypomastigotes were grown in cultures of Va-13 cells, an SV-40 virus transformed WI-38 human lung cell line obtained from the American Type Culture Collection. Va-13 cells were infected by exposure to bloodstream trypomastigotes overnight. The tissue cells were rinsed, resupplied with medium and continuously subcultured with the periodic addition of uninfected tissue cells. Yield was about 5 ×

10^7 culture-form trypomastigotes per 75 cm^2 flask per day after the culture was established. Culture-form trypomastigotes were pelleted by centrifugation twice in PSG ($I = 0.217$). Remaining host cells and cellular debris were removed by passage over a DEAE-cellulose column described above.

Amastigotes were harvested from infected Va-13 cells as follows. Infected monolayers were washed five times with cold phosphate-buffered saline pH 7.4 (PBS), the cells were removed by scraping and resuspended in 5 ml PBS. DNAse (100 $\mu g\ ml^{-1}$), RNAse A (100 $\mu g\ ml^{-1}$), and phenylmethylsulfonylfluoride (2 mM) were added and the cells disrupted for 60 s at full speed in a stainless steel Eberbach micro blender. Amastigotes were recovered by centrifugation onto a 20% isotonic metrizamide cushion [13]. The amastigotes were removed and resuspended in 5 ml PBS containing DNAse (50 $\mu g\ ml^{-1}$), RNAse (50 $\mu g\ ml^{-1}$), and phenylmethylsulfonylfluoride (2 mM) and allowed to set on ice for 10 min. The cells were then recentrifuged onto a metrizamide cushion, recovered and washed with cold PBS.

Staphylomastigotes were obtained by transformation of culture-form trypomastigotes in ML-15H [10]. Staphylomastigote masses from 5 day cultures were separated from culture-form trypomastigotes by centrifugation at $50 \times g$ for 1 min. The cell pellet was resuspended in 10 ml ML-15H and washed twice with cold PBS. Staphylomastigotes obtained by this procedure were contaminated with less than 1% culture-form trypomastigotes. Subculturing into ML-15H at 3 day intervals resulted in log phase growth with less than 1% contamination with epimastigotes or trypomastigotes.

Epimastigotes were grown in THOSMEN [14] at 28°C without shaking. Log phase cells were greater than 99% epimastigotes. Cells were harvested at $800 \times g$ at 4°C.

Metacyclic trypomastigotes were produced by transformation of epimastigotes by the procedure of Sullivan [15] and were separated from remaining epimastigotes by the procedure of Al-Abbassy et al [16]. Cells were greater than 98% metacyclics after these procedures.

Iodination. Cell surface proteins were radiolabeled with $Na^{125}I$ by a modification of the Iodogen procedure of Markwell and Fox [17]. Cells were washed three times in PBS containing 1% glucose, and resuspended in 0.9 ml PBS containing 1% glucose. Iodogen (10 μg) was dissolved in dichloromethane and dried in a glass test tube containing about fifteen 4–5 mm glass beads. $Na^{125}I$ (1.0 mCi) was added to the 0.9 ml cell suspension and the cells were incubated with the Iodogen coated glass beads for 20 min at room temperature with gentle shaking. By phase contrast microscopy cells were normal and those with a flagellum were motile. The cells were washed 3 times in PBS containing 1% glucose and solubilized in membrane dissolving buffer on ice for 20 min with occasional gentle mixing. Nuclei and particulate matter were removed by successive centrifugation of 2 000 and 15 000 rpm for 10 min each at 4°C. The supernatant was removed and stored in aliquots which were either used immediately or frozen at −70°C with glycerol added to a final 10% concentration.

Protein concentration was determined with a Bio-Rad Protein Assay Kit. The micro assay was performed according to the manufacturer's instruction.

Isoelectric focusing, first dimension. Electrophoresis was performed according to O'Farrel et al [7] with the following modifications. Four tube gels (10 cm × 0.5 cm) were prepared as follows: 1.5 g urea, 340 µl 10% Triton X-100, 400 µl of a 30% acrylamide-5.3% *N,N'*-diallyltartardiamide solution and 15 µl 10% ammonium persulfate were added to 720 µl H_2O. The solution was degased and 282 µl of Pharmalyte 3-10 was added. The gels were overlayed with 8 M urea and polymerized 1 h. Samples containing 100 000–200 000 cpm were mixed with 5 µl (5µg each protein) of BRL High Molecular Weight Marker Kit and solid urea added to 8 M. The sample was loaded on the gel and overlayed with 4 M urea and electrofocused at 8°C, 500 V for 14 h then increased to 1 000 V for 1 h. The upper chamber (cathode) contained 0.01 M ethylenediamine and the lower chamber (anode) contained 0.01 M iminodiacetic acid. After focusing the gels were removed and equilibrated in 2.3% SDS, 63 mM Tris-HCl, 5% 2-mercaptoethanol, 10% (w/v) glycerol, 0.01% bromophenol blue for 20 min at room temperature and either loaded immediately onto a second dimension gel or frozen at -70°C and used within 2 weeks.

SDS gels, second dimension. A 10% acrylamide gel (1.5 mm × 10 cm × 14 cm) was prepared as follows: the lower gel (6.4 ml lower gel buffer [7], 8.5 ml of a 25.8% acrylamide-4.2% *N,N'*diallyltartardiamide solution, 10.6 ml H_2O, 150 µl 10% ammonium persulfate, and 15 µl TEMED) was poured, overlaid with sec-butanol saturated with one quarter strength lower gel buffer and polymerized overnight at room temperature. The upper gel solution (2.5 ml upper gel buffer [7], 1.5 ml of a 30% acrylamide-1.5% *N,N'*-diallyltartardiamide solution, 6 ml H_2O, 30 µl 10% ammonium persulfate, and 10 µl of TEMED), was layered on the lower gel, and polymerized for 1 to 2 h. The equilibrated isoelectric focusing gel was layered on the second dimension gel, sealed in place with a 1% agarose solution, and electrophoresed at 20 mA at 4°C until the tracking dye ran off the bottom.

One dimensional SDS gels. Lower and upper gels were made as described above for second dimension gels. Samples containing 50 000–100 000 cpm were mixed with an equal volume of 2 × SDS sample buffer and boiled 3 min before loading. Gels were electrophoresed as described above.

Fixing, staining, destaining and autoradiography. Gels were fixed and stained overnight in 25% isopropanol, 10% acetic acid, 0.025% Coomassie Brilliant Blue R; destained in 10% isopropanol, 10% acetic acid; dried using a Bio-Rad gel dryer with heat and vacuum and autoradiographed using XRP-5 film at -70°C with an intensifying screen.

Use of internal standards in 2 dimensional gels. Three proteins supplied in the BRL

High Molecular Weight Protein Marker Kit had p*I* values within the range of the isoelectric focusing gel: phosphorylase *b* 92 500 M_r, p*I* = 6.9; bovine serum albumin 68 000 M_r, p*I* = 5.8; and ovalbumin 45 000 M_r, p*I* = 5.4. The addition of marker proteins at 5 μg each did not interfere with running of the gel sample and each was detectable by staining (Fig. 1). These provided internal standards which, when compared to the autoradiograph of the gel, allowed an accurate assignment of p*I* and M_r values to spots present on the autoradiographs. Accordingly, it allowed a direct comparison of autoradiographs of different samples.

Western blotting. Transfer of proteins to nitrocellulose membranes was performed according to Burnette [18] but the concentration of Tris was increased from 25 mM to 250 mM. After transfer filters were treated as described by Burnette [18] and reacted at 37°C overnight with a 1:10 dilution in 10 mM Tris, 0.9% NaCl, 5.0% BSA, 0.02% NaN_3, pH 7.4, of serum from a hyperimmunized mouse. Filters were rinsed and reacted at 37°C overnight with 5 ml rabbit anti-mouse IgG (Miles - Yeda Ltd) in 10 mM Tris, 0.09 NaCl, 5.0% BSA, 0.02% NaN_3, pH 7.4, at a final concentration of 0.3 μg antibody per ml. Filters were rinsed and reacted with $Na^{125}I$ labeled protein A in 10 ml of 10 mM Tris, 0.9% NaCl, 5.0% BSA, pH 7.4, (1×10^5 cpm ml^{-1}; spec. act. = 5 Ci g^{-1}) for 20 min at room temperature. Filters were rinsed and exposed at -70°C.

Hyperimmunized mice. Mice (C57BL/6 × DBA/2) F_1 [19] were inoculated with 10^3 bloodstream trypomastigotes. These mice survived the infection and were boosted with 10^3 live bloodstream trypomastigotes every 2 wks for 6 wks. Serum was obtained 12 days after the last injection.

RESULTS

Internal standards. In an attempt to assign more accurately p*I* and M_r values to proteins detected by two dimensional gel electhrophoresis, a system of internal standards was employed. As shown in Fig. 1, the position in the 2nd dimension gel of three of the proteins used as internal standards was logarithmically related to the apparent molecular weights of these proteins and linearly related to their isoelectric points. Thus, by employing internal protein standards both p*I* and M_r values can accurately be assigned to the surface proteins detected by two dimensional gel analysis.

Analysis of iodinated cell surface proteins. One and two dimensional gel electrophoretic profiles of radioiodinated cell surface proteins of the six different stages of *T. cruzi* are shown in Fig. 2. The apparent molecular weights and p*I* values of the individual protein species identified by this approach are presented in Table I. The major surface proteins which were detected by this method were relatively acidic with p*I* values in the range of 5.2 to 6.6. As shown in Fig. 2 the major surface components of the blood-

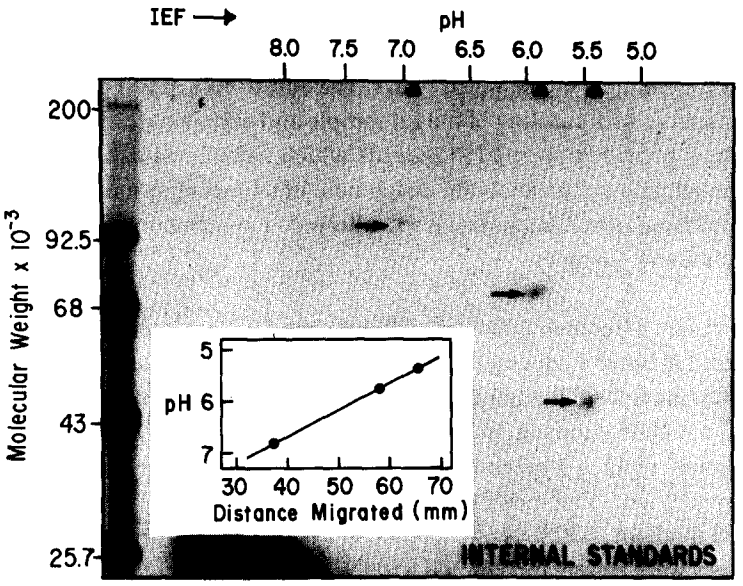

Fig. 1. Two dimensional gel profile of internal protein standards stained with Coomassie Blue. Inset shows the relationship between distance migrated by phosphorylase *b* (92 500 M_r), bovine serum albumin (68 000 M_r) and ovalbumin (43 000 M_r) in the first dimension (IEF) and their respective p*I* values (▲).

stream and culture-form trypomastigotes were quite similar. Both forms possess several major proteins of 90 000 M_r with p*I* values in the 5.5 to 6.6 range. This observation is in agreement with previous studies which have identified a 90 000 M_r protein on the surface of one or both of these forms [2–6]. However, this is the first indication that the single band at 90 000 M_r, previously seen only on one dimensional gel profiles, represents multiple protein species with the same molecular weight. Several surface components present on the bloodstream trypomastigotes are absent on the culture-form trypomastigotes. In particular, a major surface component of 68 000 M_r and several proteins of >100 000 M_r were observed only on the bloodstream trypomastigotes. Considering the 68 000 M_r protein's molecular weight and p*I*, it is likely that this component is bovine serum albumin introduced during the isolation of the bloodstream trypomastigotes. It is also likely that several of the >100 000 M_r proteins represent contaminants that arise from platelets which remain in the preparation [3]. A major protein of 90 000 M_r was also observed on the staphylomastigotes, a form which arises by transformation of either bloodstream or culture-form trypomastigotes in acellular culture conditions to an amastigote like form capable of cellular division [10]. However, unlike bloodstream and culture-form trypomastigotes only one protein was observed at 90 000 M_r by two dimensional gel analysis.

Surprisingly, the metacyclic trypomastigote which, like the bloodstream and culture-form trypomastigotes, is an invasive form of the parasite, showed a surface protein pattern quite distinct from that of either of the latter forms. In fact, the profile of the

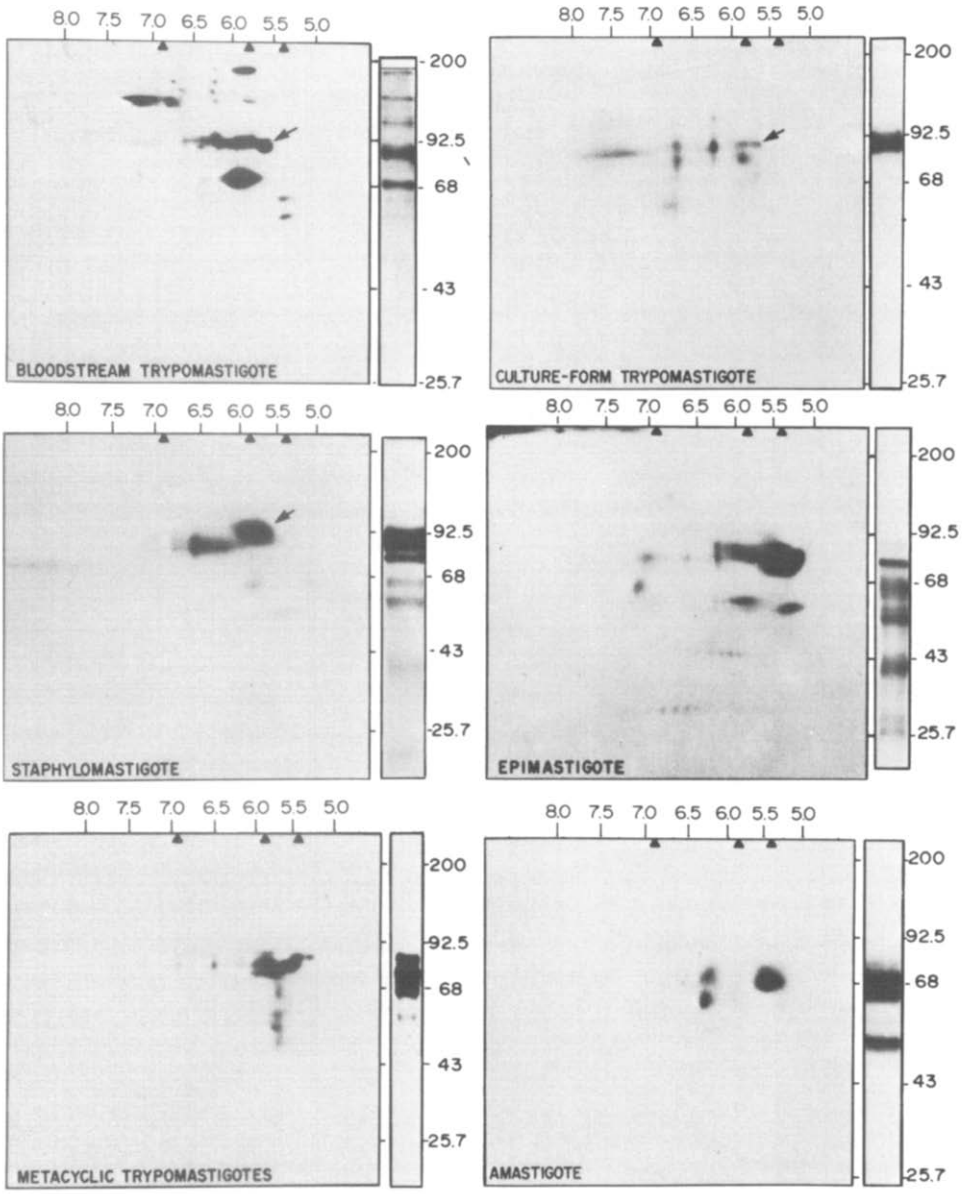

Fig. 2. One and two dimensional gels of ^{125}I-labeled surface proteins of various forms of *T. cruzi*. ▲ designates the positions of the internal standards in the second dimension gel as determined by staining with Coomassie Blue (see Fig. 1). The arrow indicates the position of the protein that reacted with antibody (See Fig. 4).

TABLE I - Cell surface proteins on six stages of *T. cruzi*

Stage	Molecular mass (kDa)	pI
Bloodstream trypomastigotes	165	5.8
		135 6.7
	135	6.8–7.2
	* 90	5.5
	* 90	5.7–6.0
	* 90	6.2
	68	5.8
	58	5.4
	51	5.4
Culture-form trypomastigotes	135	7.2
	* 90	5.5
	* 90	5.8
	* 90	6.2
	* 90	6.6
	86	7.2–7.7
	82	5.8
	82	6.6
Staphylomastigotes	* 90	5.5
	80	6.0–6.5
	68	5.4
Epimastigotes	77	5.4
	76	5.2–5.4
	* 72	5.2–5.4
	57	5.8
	55	5.2
	43	5.8–6.1
	33	6.5–6.8
Metabolic trypomastigotes	85	5.4
	77	5.8
	76	5.6–5.7
	76	6.5
	76	6.9
	* 72	5.7
	66	5.6
	55	5.6
	50	5.6
Amastigotes	71	6.2
	68	5.4
	62	6.2

*Major proteins.

metacyclic trypomastigote surface proteins was remarkably similar to that of the epimastigote form. In both the epimastigote and metacyclic trypomastigote several major surface proteins were clustered in the range of 72 000 to 77 000 M_r with pI values between 5.2 and 6.9. The metacyclic trypomastigote profile did, however, show a major surface component at 85 000 M_r which is absent from the epimastigote profile. In neither the metacyclic trypomastigote nor the epimastigote profile were we able to detect a 90 000 M_r protein similar to that observed in the bloodstream trypomastigote and culture-form trypomastigote profiles.

Identification of immunodominant surface antigen. Although Nogueira et al. [3] also reported the absence of a 90 000 M_r protein on the surface of the epimastigote and metacyclic trypomastigote, several other laboratories have clearly identified a major 90 000 M_r protein on the surface of the epimastigote [2,5,6]. The latter studies are particularly relevant since, collectively, they demonstrate that the 90 000 M_r surface polypeptide on bloodstream trypomastigote, culture-form trypomastigote and epimastigote is the immunodominant surface component recognized by antibodies in human IgG obtained from Chagasic patients, serum from mice infected with *T. cruzi* and rabbit anti-trypomastigote serum. One notable possibility that could account for the variance between our results and those of other investigators is that the 90 000 M_r protein is present in the epimastigote and metacyclic trypomastigote but is refractory to labeling by the conditions employed in this study. This possibility is directly amenable to investigation by Western blot analysis. Each of the six forms of *T. cruzi* were individually solubilized and the lysates were subjected to one dimensional gel

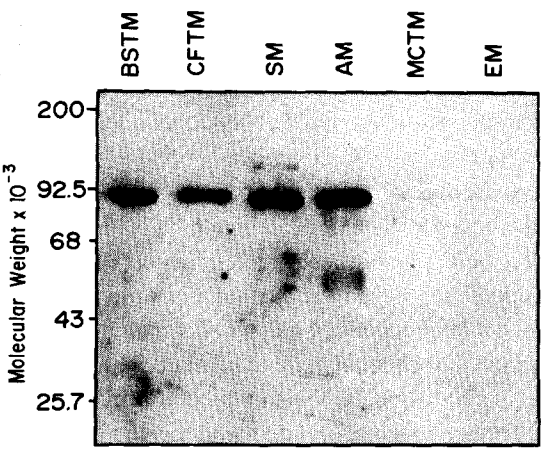

Fig. 3. Western blot analyses of the unlabeled proteins of different forms of *T. cruzi*. 10 µg of protein from each form was fractionated by PAGE and Western blotted to nitrocellulose. The blot was reacted with hyperimmunized mouse sera and bound antibody was detected with rabbit antimouse sera and ^{125}I-labeled protein A. Bloodstream trypomastigote (BSTM); culture form-trypomastigote (CFTM); staphylomastigote (SM); amastigote (AM); metacyclic trypomastigote (MCTM); epimastigote (EM).

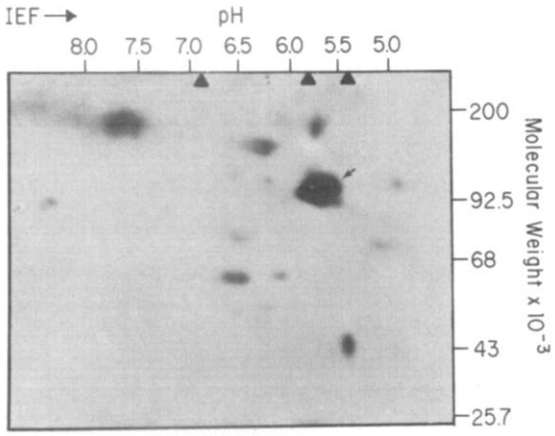

Fig. 4. Western blot analyses of the unlabeled proteins of the bloodstream trypomastigote form of *T.cruzi*. 100 μg of protein was electrophoresed in two-dimensions (IEF and SDS-PAGE), Western blotted to nitrocellulose, and reacted with hyperimmune mouse serum as in Fig. 3. The arrow indicates the major antigens.

electrophoresis. The fractionated proteins were transferred to nitrocellulose by electroelution [18] and reacted with hyperimmune mouse serum. As shown in Fig. 3, *T. cruzi* specific antibodies present in the sera of infected mice reacted strongly with a protein(s) of 90 000 M_r in four of the six forms of the parasite. No reaction, however, was observed with either the epimastigote or metacyclic trypomastigote forms. This result is consistent with the surface protein labeling studies and further suggests that this antigen is not present in either the epimastigote or metacyclic trypomastigote.

Since the two dimensional gel profiles of the bloodstream trypomastigotes and culture-form trypomastigotes identified several proteins of 90 000 M_r, it was desirable to determine how many of these proteins were recognized by antibodies in the hyperimmune mouse sera. As shown in Fig. 4, a Western blot analysis of a two dimensional gel of bloodstream trypomastigotes indicates that two major proteins of 90 000 M_r and p*I* 5.6–5.8 were reactive while the 90 000 M_r protein at p*I* 6.2 (Fig. 2) was not recognized by the antiserum. Interestingly, one of these proteins possesses similar M_r and p*I* values to the 90 000 M_r protein observed on the surface of staphylomastigotes by both surface labeling studies and Western blot analysis. If further analysis confirms that the 90 000 M_r antigen on the staphylomastigotes is identical to one of those present on trypomastigotes, the comparative ease with which staphylomastigotes may be cultured would make this form a good candidate for use in the isolation of the antigen in large quantities.

DISCUSSION

In general, our results are in agreement with those of Noguiera et al. [3,4], who have

identified a 90 000 M_r antigen on the surface of bloodstream trypomastigotes but have not detected a similar antigen on the surface of either the epimastigote or metacyclic trypomastigote. Both results are, however, at variance with reports on the presence of a 90 000 M_r antigen of the surface of epimastigotes [2,5,6]. Although the basis for these differences has not yet been determined, several possible explanations have been examined.

Zingales et al. [6] suggest that the surface proteins of *T. cruzi* are more amenable to detection when ^{131}I rather than ^{125}I is employed as the radionuclide. Whether or not this is the case, we do not favor the possibility that the 90 000 M_r peptide is present on the surface of the epimastigote but is refractory to detection by the radioiodination procedures used in our studies. The results of the Western blot analysis (Fig. 3) clearly show that the 90 000 M_r antigen is found in four of the stages examined but is absent in the epimastigote and metacyclic trypomastigote. Since this approach does *not* rely on the cellular location of the antigen for detection, it strongly argues against the presence of the 90 000 M_r polypeptide as an abundant antigen either on the surface or as an internal protein in the epimastigote and metacyclic trypomastigote forms.

We have also examined the possibility that the appearance of the 90 000 M_r surface antigen on epimastigotes depends on cell growth (i.e. log vs stationary) and/or culture conditions (Beard and Manning, manuscript in preparation). Although qualitative and quantitative differences in peptides present on the surface of epimastigotes of the Y and Peru strain were observed, no 90 000 M_r surface peptide was detected by these studies. We have also isolated the surface proteins of both the epimastigote and culture-form trypomastigote of the Peru strain by iminobiotin-avidin affinity chromatography ([20] and Beard and Manning, manuscript in preparation). In these preparations the 90 000 M_r antigen represents the major surface protein from the culture-form trypomastigote but is not detected in the epimastigote.

Morel et al. [21] report the interesting finding that individual clones of laboratory stocks of both the Y and C1 strains of *T. cruzi* exhibit heterogeneity in their K-DNA restriction endonuclease patterns. They also report similar results for putative Y strain cultures obtained from different laboratories. In view of these results, it is conceivable that the Y and C1 strains used by the various investigators for surface protein analysis exhibit different biological properties; one of which may be the expression of the gene sequence encoding the 90 000 M_r protein in the epimastigote stage. Studies which will test this possibility are in progress.

In conclusion, we have presented evidence that the surface protein patterns of the bloodstream and culture-form trypomastigotes are quite similar with both forms having several 90 000 M_r surface peptides not all of which are recognized by hyperimmune mouse serum. The epimastigote and metacyclic trypomastigote surface protein patterns are also comparable and are distinctly different from those of the bloodstream and culture-form trypomastigotes. The major surface components on the epimastigote and metacyclic trypomastigote are a group of proteins of 72–77 000 M_r. Proteins of similar M_r and p*I* were not observed in the bloodstream or culture-form

trypomastigotes. In addition, the 90 000 M_r immunoreactive protein(s) present on the bloodstream, culture-form trypomastigotes, staphylomastigotes and amastigotes could not be detected on epimastigotes or metacyclic trypomastigotes by either the surface labeling techniques or Western blot analysis with hyperimmune mouse serum.

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