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IN VITRO DIFFERENTIATION OF *TRYPANOSOMA CRUZI* UNDER CHEMICALLY DEFINED CONDITIONS

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Metacyclic trypomastigotes of *Trypanosoma cruzi* have been obtained in chemically defined axenic culture. The differentiating medium, composed of artificial triatomine urine supplemented with proline, allows high yields of metacyclic trypomastigotes after 72-h incubation of *T. cruzi* cells at 27°C. Morphological differentiation of the parasites is gradual under these chemically defined conditions and is preceded by the expression of stage-specific polypeptides. The yield of in vitro-induced metacyclic trypomastigotes depends upon the age of the epimastigote culture, the size of the inoculum and the depth of the medium. Metacyclic trypomastigotes differentiated in vitro from the Dm 28c clone of *T. cruzi* are both resistant to complement lysis and to macrophage digestion. They are able to infect mice with an efficiency similar to that obtained for natural metacyclic trypomastigotes obtained from triatomine excreta.

Key words: *Trypanosoma cruzi*; Metacyclogenesis; In vitro differentiation

INTRODUCTION

The study of trypanosomatid morphogenesis provides useful information about differentiation in unicellular eukaryotes because distinct morphological types alternate during the life cycle. The transformation of epimastigotes into metacyclic trypomastigotes at 27°C (metacyclogenesis) is a crucial step during the *Trypanosoma cruzi* life cycle. This process occurs naturally in the digestive tract of triatomine insect

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TAU, artificial triatomine urine; TAUP, artificial triatomine urine supplemented with 10 mM proline; TAUS, artificial triatomine urine supplemented with 10% new born calf serum; EDTA, ethylenediaminetetraacetic acid; LIT, liver infusion tryptose medium.

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vector. In addition, the study of *T. cruzi* metacyclogenesis deserves special interest since this parasite is the etiological agent of American trypanosomiasis or Chagas' disease [1,2].

The establishment of experimental conditions for the axenic culture of *T. cruzi* made possible the in vitro study of metacyclogenesis [3]. Several workers obtained *T. cruzi* metacyclics in non-defined media [3–10], on partially defined media [11,12], or in chambers subcutaneously implanted in mice [13]. However, the interpretation of the results has been rendered difficult since the differentiation was sometimes accompanied by a concomitant cell replication event.

We have recently described a semi-defined medium allowing the transformation of cloned *T. cruzi* Dm 28c strain epimastigotes into metacyclic trypomastigotes in the absence of cell multiplication [14]. This was accomplished by submitting epimastigote cells to a nutritional stress. This experimental condition allowed a metabolic synchronization of the cells in such a way that further incubation in a relatively poor medium allowed the transformation of epimastigotes into metacyclic trypomastigotes.

The possibility of obtaining large amounts of metacyclic trypomastigotes in vitro with similar biological properties to insect-derived forms would aid studies with *T. cruzi*. In particular, studies on differential gene expression, molecular mechanisms of morphogenetic regulation, properties of stage-specific surface antigens and stage- and strain-specific metabolic pathways would greatly benefit.

In the present study we describe the use of a chemically defined differentiating medium in obtaining high yields of *T. cruzi* metacyclic trypomastigotes. We have investigated the kinetics of appearance of trypomastigote biological properties as well as protein synthesis and expression of stage-specific surface antigens during the in vitro metacyclogenesis of *T. cruzi* Dm 28c.

MATERIALS AND METHODS

Parasites. *T. cruzi* Dm 28c was obtained and kept in the laboratory as previously described [14,15]. *T. cruzi* strains Dm 30, EP and PP are normally maintained in our laboratory, while strains CL, G, and two samples of Y strain from different sources were kindly provided by Drs. E. Chiari (Universidade Federal de Minas Gerais, Brazil), N. Yoshida (Escola Paulista de Medicina, Brazil), W. Colli (Universidade de São Paulo, Brazil) and J. Dvorak (NIH, Bethesda, U.S.A.), respectively.

In vitro differentiation. *T. cruzi* cultures grown in liver infusion tryptose (LIT) medium at 27°C and containing approximately 100% of epimastigotes at the end of the exponential phase were centrifuged at 10 000 $\times g$ for 15 min at 10°C, resuspended in artificial triatomine urine (TAU) (190 mM NaCl, 8 mM phosphate buffer pH 6.0, 17 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$), and incubated for 2 h at room temperature. The parasites were diluted to a final concentration of 5×10^6 parasites ml^{-1} in TAU supplemented with 2.5% (v/v) sodium bicarbonate 1.4%, 500 units penicillin ml^{-1}, 10

mM L-proline (TAUP medium) and incubated at 27°C in tightly closed culture flasks. The flasks had a liquid depth not exceeding 10 mm and were incubated without agitation for different times according to the experimental schedule.

In vivo labelling of parasites with [^{35}S]methionine. 1 × 10^7 parasites incubated in TAUP for different times were centrifuged at 12 000 × *g* for 3 min at room temperature and washed in TAU. The cells were incubated for 120 min in 1 ml of Hanks' solution supplemented with 10 mM Hepes pH 7.4 and 100 μCi [^{35}S]methionine (New England Nuclear, 1 045 Ci mmol^{-1}). Labelled parasites were centrifuged for 3 min at 12 000 × *g*, resuspended in 0.5 ml of lysis solution (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetate (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM iodoacetamide, 1% Nonidet-40 (NP-40)) homogenized for 3 min at room temperature and centrifuged for 10 min at 12 000 × *g*. The supernatant was immediately processed for immunoprecipitation or electrophoresis, as previously described [14].

Preparation of antisera. Epimastigote and trypomastigote stage-specific antisera were prepared as previously described [14]. Antiserum directed against metacyclic trypomastigote proteins stimulated by complement [16] (PC antiserum) was prepared in rabbits. 1.3 g of Dm 28c metacyclic trypomastigotes from TAUS medium [14] were resuspended in 30 ml of Hanks' solution and incubated with 30 ml of fresh guinea pig serum at 37°C for 45 min. Parasites resistant to complement lysis were centrifuged at 10 000 × *g* for 15 min at 4°C. The pellet was resuspended in 1 l of Hanks' solution containing 20 mM Hepes pH 7.4, 10 mM methionine and incubated for 2 h at 27°C. The parasites were centrifuged as above and lysed by stirring for 3 min in 20 ml of 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 2 mM iodoacetamide, 1% NP-40 at room temperature. The supernatant from lysed cells was immunoprecipitated with 15 ml of globulin fraction of the epimastigote antiserum (2.7 mg protein ml^{-1}) [14] and the supernatant from this immunoprecipitation was lyophilized and used as antigen for rabbit immunization.

New Zealand rabbits weighing 3 kg were inoculated with 18 mg of the antigen in incomplete Freund's adjuvant by 20 subcutaneous injections. After 8 days the animals were injected intramuscularly with the same amount of antigen. Two weeks later the rabbits were submitted to three intravenous injections of 1.8 mg of antigen in saline solution at 48-h intervals. After 3 weeks the animals were bled and the immune response was determined by indirect immunofluorescence [17] with complement-treated metacyclic trypomastigotes, displaying a titre of 1:1 600.

Polyacrylamide gel electrophoresis (PAGE) and fluorography. Proteins were analyzed by electrophoresis on linear gradients of 10–15% sodium dodecyl sulfate (SDS)-polyacrylamide gels [18]. Gels were treated for fluorography with Enhance (New England Nuclear Inc.) and the dried gels were autoradiographed using Sakura Medical Imaging films (Sakura, Japan) and Kodak-X-O-matic regular intensifying screens.

Resistance to complement mediated lysis. Parasites were resuspended either in Eagle's minimum essential medium or in TAUP medium in a ratio of 5×10^6 parasites ml^{-1}. 10 μl of fresh guinea pig serum was added to 50 μl of parasite suspension and the cells were incubated at 37°C for 45 min. The motile cells were counted in a Neubauer hematocytometric chamber and compared with controls similarly processed but with heat-inactivated (56°C, 60 min) guinea pig serum.

Resistance to macrophage digestion. To 10^6 parasites in 500 μl of TAUP medium we added 500 μl of eliciated macrophages in RPMI-1640 medium in a ratio of 8 parasites per macrophage. After incubation for 12 h at 37°C the cells were fixed and stained with Giemsa and 300 macrophages were randomly selected for the determination of infectivity and morphology of the parasites.

Metacyclic trypomastigote infectivity to animals. Male albino mice weighing approximately 12 g were inoculated subcutaneously either with *T. cruzi* Dm 28c in vitro-induced metacyclic trypomastigotes or with *T. cruzi* Dm 28c metacyclic trypomastigotes from triatomine urine. The parasitemia was followed from the fourth day of inoculation by fresh blood examination [19].

RESULTS

The differentiating medium. Various L-amino acids were individually assayed in TAU in order to determine a minimal medium capable of supporting *T. cruzi* metacyclogenesis. The results shown in Table I indicate that from the 10 L-amino acids assayed only L-proline supported the differentiation of epimastigotes to metacyclic trypomastigotes at high rates and with low cell damage. Addition of leucine, valine, alanine, glycine, histidine and cysteine resulted in cell lysis after 24 h of incubation in TAU. Other amino acids resulted in very poor yields of differentiation (phenylalanine, methionine and aspartic acid) with considerable cell lysis after 72 h of incubation. Variations in the concentration of proline from 5 to 50 mM did not affect the final percentage of morphological metacyclic trypomastigotes. Other factors affecting the yield of in vitro-induced metacyclic trypomastigotes, such as size of the inoculum and the oxygen tension (as inferred by the depth of the culture medium) were determined. The best results were obtained for an inoculum of 3×10^6 cells ml^{-1} and a liquid depth of 10 mm. Values above these resulted in poor yields due to cell lysis (results not shown).

Various strains of *T. cruzi* were assayed in the differentiating medium. The differentiation rate varied according to the strain (Table II). Some strains did not show high yields of metacyclic trypomastigotes in a preliminary assay. However, after the treatment of the differentiated cells with fresh guinea pig serum, followed by the culture of the remaining parasites in LIT medium and a second cycle of incubation in TAUP medium, the parasites displayed differentiation rates comparable to those obtained for

TABLE I

Effect of different substrates on in vitro differentiation of *T. cruzi* Dm 28c

Substrate[a]	% Metacyclics	Parasites mm^{-3}	
		Inoculated	Recovered
TAU	[b]	1596 ± 101	
Hanks'	50 ± 12	1587 ± 200	89 ± 81
TAUS	30 ± 4	1969 ± 101	1963 ± 331
LIT-B	4 ± 1	780 ± 118	1954 ± 353
L-Leucine	[b]	1959 ± 101	
L-Valine	[b]	1959 ± 101	
L-Phenylalanine	0	1959 ± 101	306 ± 181
L-Alanine	[b]	1959 ± 101	
L-Glycine	[b]	1959 ± 101	
L-Histidine	[c]	1959 ± 101	
L-Cysteine	[b]	1959 ± 101	
L-Methionine	0	1959 ± 101	261 ± 173
L-Aspartic acid	25 ± 8	2963 ± 168	461 ± 126
L-Proline	86 ± 5	2963 ± 168	2109 ± 347

[a] The concentration of the amino acids was 10 mM in TAU.
[b] No viable parasites detected after 24 h.
[c] No viable parasites detected after 48 h.

Dm 28c (Table II). This suggests that some strains must be conditioned to differentiate in TAUP. The time necessary for attaining the maximum level of differentiation varies for different strains of *T. cruzi* (Table II).

Protein synthesis during differentiation. The analysis of the total proteins synthesized during differentiation of *T. cruzi* Dm 28c in TAUP shows that differences in the polypeptide profile can be observed within the first 24 h of differentiation (Fig. 1, lane E). A group of polypeptides with M_r between 80000 and 85000 have their synthesis increased in relation to the preceding times of differentiation (Fig. 1, lanes A–D) continuing until 72 h of differentiation (Fig. 1, lane I) after which little modification occurs. On the other hand, polypeptides displaying M_r of 38000, 47000 and 69000 have their expression reduced as the differentiation progresses and are almost absent after 72 h of incubation in TAUP. Similarly, polypeptides of M_r greater than 100000 have their synthesis reduced as the cells transform from epimastigotes to metacyclic trypomastigotes. The comparison of the proteins synthesized by epimastigotes (Fig. 1, lane A) and metacyclic trypomastigotes (Fig. 1, lane K) points to the higher complexity of the non-infective form of *T. cruzi*. Epimastigotes synthesize polypeptides, under the chemically defined conditions employed, of M_r 150000, 135000, 125000, 105000, 90000, 82000, 72000, 63000, 60000, 48000, 45000, 41000, 37000 and 29000, while

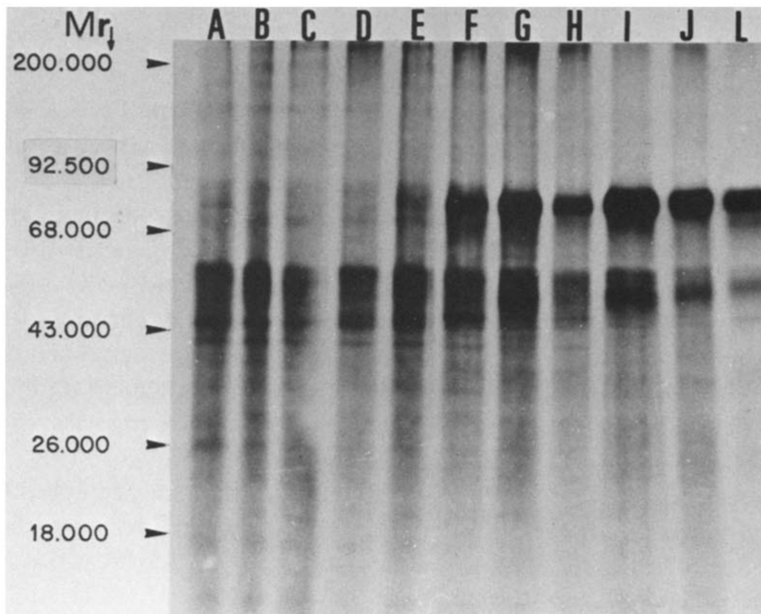
TABLE II

Differentiation of distinct *T. cruzi* strains in TAUP medium

Strain	Origin	% Metacyclics	
		1st assay	2nd assay[a]
Dm 28c	Opossum	87 ± 4 (3 days)[b]	90 ± 2 (3 days)
Dm 30	Opossum	50 ± 2 (6 days)	80 ± 3 (6 days)
EP	Human	30 ± 5 (6 days)	87 ± 5 (6 days)
PP	Human	17 ± 2 (6 days)	87 ± 5 (6 days)
CL	Triatomine	7 ± 5 (6 days)	72 ± 9 (6 days)
G	Opossum	70 ± 12 (6 days)	75 ± 5 (6 days)
Y	Human	12 ± 6 (5 days)	60 ± 7 (5 days)
Y	Human	4 ± 1 (5 days)	70 ± 11 (5 days)

[a] The differentiation was induced from epimastigotes which had been derived from metacyclic trypomastigotes produced in the first assay.

[b] Time necessary for obtaining the indicated amount of metacyclic trypomastigotes.



metacyclic trypomastigotes synthesize six polypeptides common to the epimastigote stage (90 000, 82 000, 72 000, 63 000, 60 000 and 41 000) and three stage-specific polypeptides (86 000, 75 000 and 55 000).

The immunoprecipitation of the proteins synthesized during metacyclogenesis with three distinct antisera confirms our previous results that the differentiation in terms of stage-specific gene expression products precedes the morphological differentiation [14]. Fig. 2 shows that the M_r 55 000, 51 000 and 46 000 polypeptides recognized in epimastigotes by the epimastigote antiserum (Fig. 2, lane C) become less intense after 24 h of *T. cruzi* differentiation (Fig. 2, lane G) and almost disappear after 36 h of incubation in TAUP medium (Fig. 2, lane H). On the other hand, polypeptides in the range of M_r 86 000 to 80 000 are immunoprecipitated from differentiating cells by the trypomastigote stage-specific antiserum after 36 h of incubation in TAUP (Fig. 3, lane I). This inverse correlation indicates that epimastigote antigens are replaced by metacyclic trypomastigote antigens. The change in the protein pattern is clearly seen in the

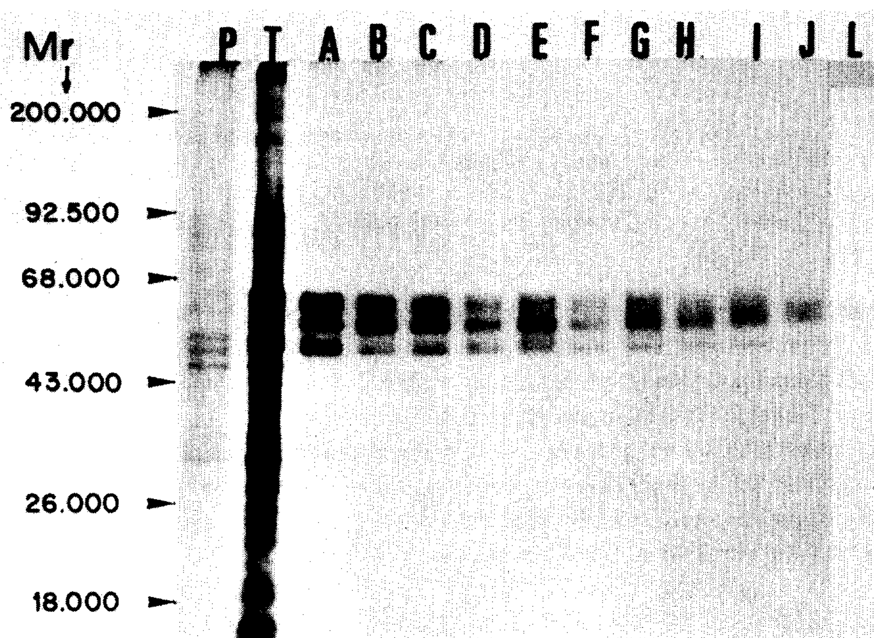


Fig. 2. PAGE of the proteins immunoprecipitated with the epimastigote antiserum from cells incubated in TAUP for different times. (A) Epimastigotes, (B) 2 h, (C) 6 h, (D) 12 h, (E) 24 h, (F) 36 h, (G) 48 h, (H) 60 h, (I) 72 h, (J) 96 h, and (L) 140 h. T refers to total proteins synthesized by epimastigotes and P to the epimastigote proteins immunoprecipitated with normal rabbit serum.

Fig. 1. PAGE of the proteins synthesized by *T. cruzi* Dm 28c cells incubated for different times in TAUP medium. (A) Epimastigotes, (B) 2 h, (C) 6 h, (D) 12 h, (E) 24 h, (F) 36 h, (G) 48 h, (H) 60 h, (I) 72 h, (J) 96 h, and (L) 140 h. Molecular weight markers are indicated on the left of the figure and were run on the same gel.

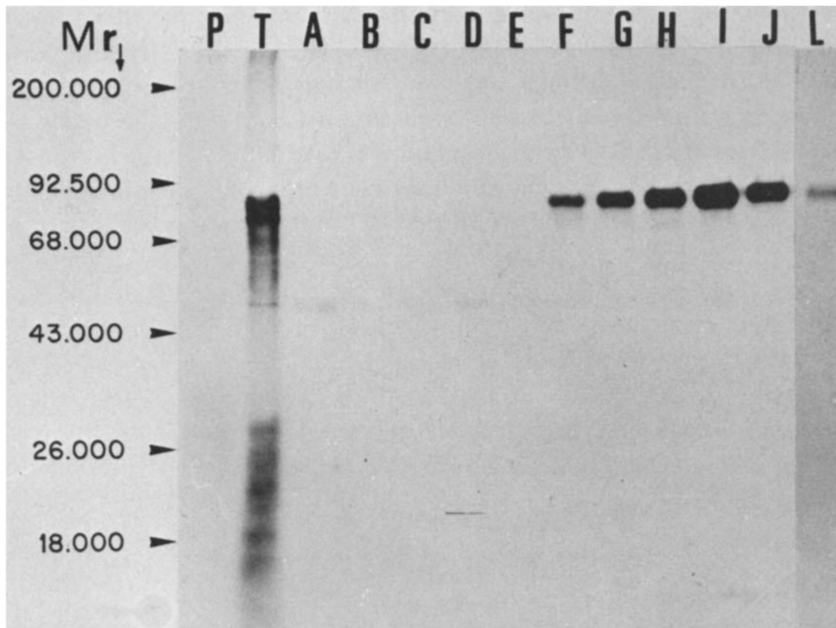


Fig. 3. PAGE of the proteins immunoprecipitated with the trypomastigote stage specific antiserum from cells incubated in TAUP for different times. (A) Epimastigotes, (B) 2 h, (C) 6 h, (D) 12 h, (E) 24 h, (F) 36 h, (G) 48 h, (H) 60 h, (I) 72 h, (J) 96 h, and (L) 140 h. T refers to total proteins synthesized by 72-h differentiating cells and P to the proteins immunoprecipitated from T by normal rabbit serum.

analysis in Fig. 4, which shows the in vivo synthesized polypeptides immunoprecipitated with PC antiserum. This antiserum recognizes polypeptides of low molecular weight in epimastigotes and high molecular weight polypeptides in metacyclic trypomastigotes. Hence, as the in vitro differentiation progresses from epimastigotes to metacyclic trypomastigotes, there is a shift in the immunoprecipitated polypeptides. Interestingly, as will be seen below, the expression of the M_r 85 000 trypomastigote stage-specific polypeptide after 36 h of differentiation coincides with the acquisition of resistance by the parasites to complement lysis.

Biological properties of differentiating cells. The acquisition by differentiating cells of the biological properties of metacyclic trypomastigotes was investigated as a function of the time of incubation of the parasites in TAUP medium. The results presented in Table III show that after 36 h of incubation in TAUP, 60% of the parasites are resistant to lysis by fresh guinea pig serum. Since there is a close relationship between the percentage of differentiated parasites and parasites resistant to complement lysis, we conclude that after 36 h of differentiation in TAUP, 100% of the morphological metacyclic trypomastigotes are resistant to complement lysis.

The ability of differentiating parasites to infect and to develop to amastigotes inside

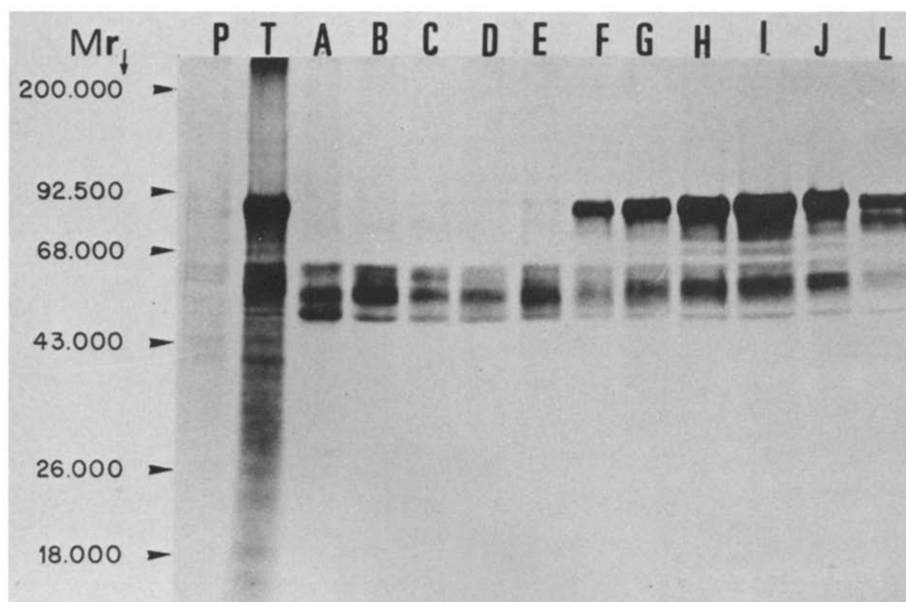


Fig. 4. PAGE of the proteins immunoprecipitated with the PC antiserum from cells incubated in TAUP for different times. (A) Epimastigotes, (B) 2 h, (C) 6 h, (D) 12 h, (E) 24 h, (F) 36 h, (G) 48 h, (H) 60 h, (I) 72 h, (J) 96 h, and (L) 140 h. T refers to total proteins synthesized by 72-h differentiating cells and P to the proteins immunoprecipitated from T by normal rabbit serum.

macrophages did not relate to the morphological changes which occurred after 48 h of incubation in TAUP (Table III). These results suggest that metacyclic trypomastigote resistance to complement lysis and the ability of *T. cruzi* to develop in macrophages are not controlled by the same set of genes.

We have tested the ability of TAUP-induced metacyclic trypomastigotes to infect mice and we have compared the results with those obtained for Dm 28c metacyclic trypomastigotes obtained from triatomine urine. The results presented in Table IV show that in vitro-induced and natural metacyclic trypomastigotes display similar biological properties. However, natural metacyclic trypomastigotes turned out to be more virulent since 40% of the inoculated mice were killed, in comparison to the 20% ratio obtained with TAUP metacyclic trypomastigotes. In both cases, the animals died 16 days after the inoculation with parasites. The animals that survived this acute phase became chronically infected and were followed for 60 days.

DISCUSSION

Several factors have been implicated in the differentiation of epimastigotes to metacyclic trypomastigotes under axenic culture conditions [20–22]. We have used a poor medium corresponding to triatomine urine and tested the effect of the addition of

TABLE III

Correlation between *T. cruzi* Dm 28c differentiation in TAUP medium and acquisition of stage-specific biological properties by metacyclic trypomastigotes

Time of differentiation (h)	% of metacyclic trypomastigotes	% infected macrophages after 72 h	% resistant to complement lysis
0	0	2	0
2	0.5 ± 0.1	2	0
6	2 ± 1	5	0
12	5 ± 2	5	0.5 ± 0.1
24	18 ± 3	10	8 ± 2
36	60 ± 5	10	60 ± 6
48	65 ± 10	60	66 ± 5
60	80 ± 8	80	74 ± 5
72	86 ± 3	80	86 ± 3
96	90 ± 2	ND	100
140	92 ± 4	ND	ND

ND, Not determined.

TABLE IV

Comparison of the infectivity of Dm 28c metacyclic trypomastigotes derived from triatomine and TAUP medium

Origin	Parasites inoculated per g of tissue[a]	Prepatency (days)	Infected/ inoculated	Dead[b]/ inoculated	Survival (days)[c]
Rhodnius prolixus	1 000	6 ± 1	10/10	4/10	60
TAUP medium	1 000	6 ± 1	10/10	2/10	60

[a] Male albino mice weighing 12 g.
[b] The animals died 16 days after inoculation.
[c] Chronically infected and killed after 60 days.

specific amino acids. Of 10 L-amino acids assayed, only proline allowed the differentiation of *T. cruzi* in artificial triatomine urine without cell damage. Concentrations of this amino acid ranging from 5 to 50 mM supported the differentiation of *T. cruzi*, while concentrations lower than 5 mM affected the reproducibility of the transformation process. As yet the role of proline is not clear. Proline might act as a trigger for the differentiation process since addition of this amino acid to LIT medium resulted in an increase in the percentage of spontaneous metacyclic trypomastigotes after 13 days of culture [22]. Proline might be an energetic source for the parasites that while under-

going physiologic stress would acquire the commitment to differentiate to metacyclic trypomastigotes. The proteins present in epimastigotes would therefore act as metabolic reserves for the differentiating cells [22].

Other factors also affect the yield of metacyclic trypomastigotes. Increase in the size of the inoculum as well as the reduction in the oxygen tension resulted in poor differentiation of the parasites. The age of the parasite culture is also important since cells taken in the beginning and in the middle of the exponential phase of growth as well as in the late stationary phase did not show high yields of differentiation (results not shown).

The metacyclogenesis of several *T. cruzi* strains in TAUP points to a process common to the species. However, in order to get comparable yields of differentiated parasites, the strain must be conditioned to the medium. As previously described, this is performed by submitting epimastigote cultures of TAUP metacyclic trypomastigotes resistant to complement lysis, to a second cycle of differentiation in TAUP (Table II). This procedure may select cell populations within the strain [23] that would be genetically committed to differentiate.

T. cruzi Dm 28c metacyclic trypomastigotes from TAUP displayed biological properties similar to those obtained from triatomine excreta in terms of their resistance to complement lysis (Table III), resistance and capacity to transform to amastigotes in macrophages (Table III) and infectivity to mice (Table IV). The slight difference in the rate of animal mortality displayed by TAUP and triatomine-derived metacyclic trypomastigotes could be due to a particular interaction of the parasite with the insect vector that would not be reproduced under axenic culture conditions.

The protein profile of metacyclic trypomastigotes from TAUP is simpler than those obtained for metacyclic trypomastigotes from TAUS medium [14] or from triatomine excreta [15]. This could be due to the fact that the differentiating medium is extremely poor in nutrients suggesting that the cells are mainly directed towards synthesizing the proteins necessary for maintaining the biological properties of the differentiation stage. The fact that *T. cruzi* cells displaying similar biological properties may express different sets of genes is in agreement with our previous results showing that metacyclic trypomastigotes synthesize different proteins according to their origin [15].

The immunoprecipitation of the proteins synthesized by differentiating cells with stage-specific antisera to *T. cruzi* shows that there is a closer relationship between morphological differentiation and the expression of stage-specific gene products in TAUP medium than in TAUS medium. This could be explained by the fact that in TAUP the cells differentiate faster and hence in a more synchronous fashion. The transition from epimastigote to metacyclic trypomastigote occurs rapidly between 24 and 36 h of incubation in TAUP. Interestingly, the appearance of a M_r 85 000 polypeptide at the end of 36 h of differentiation (Figs. 3 and 4) coincides with the acquisition by the differentiated cells of resistance to complement lysis (Table III). Since this polypeptide of M_r 85 000 is a metacyclic trypomastigote stage-specific surface antigen (manuscript in preparation) we are presently investigating the role of

this protein in conferring on the infective form of *T. cruzi* resistance to complement lysis.

The results presented above demonstrate that infective *T. cruzi* metacyclic trypomastigotes can be obtained in large amounts under chemically defined conditions. The fact that a small set of polypeptides synthesized by differentiating cells is enough to maintain the biological properties displayed by metacyclic trypomastigotes from triatomine urine indicates that the use of TAUP medium may be very useful in the study of differential gene expression during *T. cruzi* differentiation. Consequently, this medium may provide an important tool in investigating the immunoprophylaxis of Chagas' disease.

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