Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Suppl. Vol. 79 :39-44, 1984

FACTS AND HYPOTHESIS ON *TRYPANOSOMA CRUZI* DIFFERENTIATION

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The use of *Trypanosoma cruzi* as a model system for the study of cell differentiation poses a problem due to the fact that a terminal differentiation stage can not be defined for this parasite. However, differential gene expression may be studied since *T. cruzi* presents alternate morphological and functional types during its life-cycle: *Metacyclic trypomastigotes* from triatomine excreta transform to *amastigotes* inside the cells of the mammalian host, multiply as such and transform to *blood trypomastigotes* before penetrating new cells or infecting triatomines during the blood meal. Trypomastigotes ingested by the insect transform to the replicating *epimastigotes* within the invertebrate host and these again to metacyclic trypomastigotes before being released in the triatomine excreta (Brener, 1973; De Souza, 1984).

Since trypomastigotes are the infective form of the parasite, the interest in the process leading to these forms is further stimulated by the search for a more rational approach to the terapeutics and immunoprophylaxis of Chagas' disease.

We have chosen to study the transformation of epimastigotes to metacyclic trypomastigotes (metacyclogenesis). This choice was dictated by the following reasons:

a) Epimastigotes are easily obtained under axenic culture conditions (Camargo, 1964);

b) Epimastigotes are better characterized biologically (Brener, 1973) and biochemically (Gutteridge, 1981) than amastigotes;

c) Epimastigotes possess several biological properties that allow the distinction of these forms from metacyclic trypomastigotes (Brener, 1973);

d) The metacyclogenesis process may be simulated *in vitro* (Camargo, 1964; Castelanni, Ribeiro & Fernandes, 1967; Pan, 1971; Wood & Pipkin, 1969; Wood & Souza, 1976; Lanar, 1979; Chiari, 1975; Sullivan, 1983; Contreras, Morel & Goldenberg, 1985a);

e) The human infection, unless provoked by blood transfusion or accidental laboratory manipulation (Brener, 1984), is caused by the epimastigote derived metacyclic trypomastigote.

Several genes are of particular interest in *T. cruzi*:

1) The genes involved in the triggering and regulation of the metacyclogenesis process;

2) The genes that code for stage specific surface antigens;

3) The genes that code for the polypeptides involved in the acquisition of trypomastigote stage specific biological properties i.e., resistance to complement lysis (Nogueira, Bianco & Cohn, 1975) resistance to macrophage digestion (Nogueira & Cohn, 1976) and the capacity of metacyclic trypomastigotes to interiorize within mammalian cells (Zingales et al., 1982; Andrews, Katzin & Colli, 1984).

However, to study differential gene expression during the transformation of epimastigotes to metacyclic trypomastigotes *in vitro*, two major conditions should be attained: First, the development of axenic culture conditions allowing high yields and reproducibility of the differentiation of epimastigotes to metacyclic trypomastigotes. Second, the production of a homogeneous cell population in order to assure that the differences observed in the gene expression programme are not due to a selection of cell populations within the strain (Deane et al., 1984). We have circumvented both problems by developing two distinct *in vitro* differentiating media and by cloning *T. cruzi* cells.

The aim of the present article is not to review the biology of *T. cruzi*. Comprehensive reviews have been recently published (Brener, 1973; De Souza, 1984). In this article we will focus our attention on the discussion of some of the results recently obtained in our laboratory, and propose some working hypothesis centered on our experimental observations.

This investigation received financial support from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Financiadora de Estudos e Projetos (FINEP).

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T. cruzi clone Dm 28c

T. Cruzi Dm 28c was cloned by micromanipulation of an opossum isolate. The wild strain was "filtered" for some tripomastigote stage specific biological properties before cloning. These were: infection of triatomines, infection of vertebrates, penetration into *in vitro* cultured cells, resistance to macrophage digestion, resistance to complement lysis and capacity to differentiate *in vitro* (Goldenberg et al., 1984a). In order to assure the maintenance of its biological properties, the clone is re-isolated every 6 months after infection of the invertebrate and vertebrate hosts.

In vitro differentiating media

We have adapted a semi-defined medium for the *in vitro* differentiation of *T. cruzi* Dm 28c. This medium is composed of artificial triatomine urine (TAU) (190 mM NaCl, 8 mM phosphate buffer pH 6.0, 17 mM KCl, 2 mM CaCl2, 2 mM MgCl2) supplemented with 10% new born calf serum (TAUS medium) (Contreras, Morel & Goldenberg, 1985a).

A chemically defined medium was later developed based on TAUS medium. This chemically defined differentiation medium, named TAUP, is composed of TAU supplemented with 10 mM L-proline (Contreras et al., submitted). This medium was recently improved by adding 50 mM sodium glutamate, 2 mM aspartic acid and 10 mM glucose to TAUP.

The choice for L-proline was based on the fact that this is the predominant aminoacid in *R. prolixus* haemolymph and in triatomine urine (Maddrell & Gardiner, 1980). Indeed, when we assayed 10 different L-aminoacids in TAU, only L-proline promoted high yields of metacyclic trypomastigotes (Contreras et al., submitted).

The transformation of epimastigotes to metacyclic trypomastigotes

This process occurs naturally inside the insect vector midgut. A comprehensive discussion about the process of *T. cruzi* metacyclogenesis within the invertebrate host is presented by Garcia et al. in this Symposium.

Several factors have been described as stimulating *in vitro* the transformation of epimastigotes to metacyclic trypomastigotes. Some of these factors are as yet undetermined since they are present in sera (O' Daly, 1976; Sher, Crane & Kirchhoff, 1983; Contreras, Morel & Goldenberg, 1985a) or in triatomine haemolymph (Wood & Pipkin, 1969; Wood & Souza, 1976; Chiari, 1975). Other factors leading to *in vitro* metacyclogenesis are best characterized in the sense that all of them represent physiological stress for the parasites, such as variation in the pH (Fernandez & Cacerez, 1976), variation in the temperature (Pan, 1971; Bertelli, 1976), old medium (Camargo, 1964) and nutritionally poor media (Chiari, 1975; Contreras, Morel & Goldenberg, 1985a). However, the exact mechanism by which these different stresses induce the appearence of metacyclic trypomastigotes remains to be elucidated.

Metacyclogenesis in TAUS – The use of TAUS medium in the metacyclogenesis of *T. cruzi* Dm 28c resulted in a high yield (85 + 5%) of metacyclic trypomastigotes, without multiplication, after 6 days of incubation. It was observed that the differentiation process consists of firstly the expression of trypomastigote stage specific gene products and latter the morphological changes comprised in the process. While the morphological differentiation was accomplished at the end of 6 days, after 24 hours of incubation in TAUS the cells displayed a two dimensional polyacrylamide gel electrophoresis profile identical to 6 day old cells. This dissociation of the molecular and the morphological processes suggests that morphological epimastigotes might be indeed expressing metacyclic trypomastigote stage specific proteins (Contreras, Morel & Goldenberg, 1985a). Trypomastigote stage specific surface antigens are found among the proteins primarily expressed during the metacyclogenesis process and hence can be used as differentiation markers (Contreras et al., 1984). Indeed, two trypomastigote stage specific surface antigens with molecular weights of 86,000 and 78,000 are expressed by parasites after 12 hours of incubation in TAUS (Contreras, Morel & Goldenberg, 1985a).

TAUS medium, although allowing reproducibly high yields of metacyclic trypomastigotes, is not chemically defined. This characteristic imposes a limitation on this medium for some studies such as the nutritional requirements during the metacyclogenesis process, the precise characterization of the factors involved in *T. cruzi* differentiation and the isolation of metabolic mutants.

Metacyclogenesis under chemically defined conditions – The differentiation in the chemically defined TAUP medium proceeds faster than in TAUS and a closer relationship is observed among stage specific gene expression products and morphology (Contreras et al., submitted). This observation leads to the idea that a synchronization of both processes might exist. Several factors affected the yields of metacyclic trypomastigotes in TAUP. These were the size of the inoculum, the depth of the medium and the age of the epimastigote culture used for differentiation. From the molecular point of view this last criterium is quite interesting in the sense that the cells seem to show a commitment to differentiate.

Different strains of *T. cruzi*, isolated from silvatic triatomine (CL), from wild reservoirs (Dm 30, Dm 28, G) or human patients (Y, EP, PP), differentiated with comparable ratios in TAUP. However, some

of the strains needed a "pre-conditioning" in the differentiation medium (this was represented by a second cycle of *in vitro* differentiation) and the time necessary for obtaining comparable differentiation ratios (90 +/– 5%) varied for some strains (Contreras et al., submitted).

The acquisition of *T. cruzi* trypomastigote stage specific biological properties by differentiating cells was correlated with the time of incubation in TAUP and the changes in the protein profile of the parasites. The resistance to fresh guinea pig serum lysis was acquired after 36 hours of incubation in TAUP. This coincides with the expression of the Mr 86,000 and 78,000 surface antigens by differentiating cells, suggesting a close relationship between both events. After 48 hours of incubation in TAUP, the parasites acquire resistance and capacity to develop to amastigotes in macrophages. This difference in the timing necessary for the acquisition of these two trypomastigote stage specific biological properties suggests that if these properties result from trypomastigote stage specific gene expression products, different sets of genes code for each property.

The comparison between biological properties of triatomine and *in vitro* differentiated (TAUP or TAUS) Dm 28c metacyclic trypomastigotes, as evaluated by infectivity to mice, showed that although slight differences were observed in the prepatency and the mortality, the percentage of infection was identical: 100% (Contreras et al., submitted).

A molecular hypothesis to explain *T. cruzi* metacyclogenesis – It is evident that nutritional stress triggers the differentiation process within *T. cruzi*. The molecular mechanisms involved in the triggering and regulation of the metacyclogenesis process are as yet unknown. Some insight has been gained concerning the role of the nutritional stress: it provokes a shift of the epimastigote polyribosomal mRNAs reflected by a shut-off of the mRNAs that were being translated (Salles et al., 1984).

We could speculate that this shift in the translatable mRNA population would result in a selection of mRNAs coding for signal polypeptides involved in the triggering of the differentiation process. These "stress" mRNAs would be transcribed at the end of the exponential phase of growth and in the presence of a continuous nutritional stress would be selectively expressed. The epimastigote "stress proteins" could be involved in the regulation of the transcription of trypomastigote stage specific genes. Alternatively, these proteins might be involved in some particular mechanism such as genomic rearrangement.

DNA rearrangement is an important mechanism within African trypanosomes (Murphy et al., 1984). One of the important features of this genomic rearrangement could be to bring a 35 base pair mini-exon sequence to a cis-position in relation to a transcribed gene. It has been demonstrated that several mRNAs from these parasites share the same mini-exon sequence at their 5'-end (De Lange et al., 1984a; Dorfman & Donelson, 1984). In addition, it has been demonstrated that this mini-exon sequence is found in the genomic DNA of *T. cruzi* (De Lange et al., 1984b). Hence, this possibility of genomic rearrangement can not be ruled out for the moment.

One of the factors involved in some DNA repair mechanisms (and hence DNA rearrangement) is the ADP-ribosylation of chromatin proteins (Purnell, Stone & Wish, 1980; Williams & Johnstone, 1983). It has been recently suggested that *T. cruzi* amastigotes do not transform to trypomastigotes in the presence of ADP-ribosylation inhibitors (Williams, 1984). Accordingly, preliminary results show that the transformation of Dm 28c epimastigotes to metacyclic trypomastigotes is inhibited in the presence of 3-methoxy-benzinamide, a strong inhibitor of ADP-rybosyl-transferase (Goldenberg, unpublished data). Interestingly, the inhibition of *T. cruzi* metacyclogenesis was stronger when the inhibitor was added during the nutritional stress in TAU preceeding the incubation of the parasites in TAUP.

A nutritional hypothesis to explain *T. cruzi* metacyclogenesis – As discussed above, a nutritional stress triggers the metacyclogenesis process. This stress might provoke an "emergency metabolic state" where the cells would transform to one or other differentiation stage, depending upon the environmental conditions. This transition stage would be represented by a metabolic desequilibrium that could be reflected in the control of the gene expression programme (e.g., by the intracellular levels of ATP, GTP or cAMP) leading to the preferential transcription of specific sets of genes. In the persistence of the emergency metabolic state, the parasites would turn on emergency-metabolic pathways that would be specific to the trypomastigote differentiation stage. On such stage specific metabolic pathway might be the capacity of the cells to utilize aminoacids as energy sources (e.g., proline and glutamic acid in the Krebs' cycle). The utilization of aminoacids as energy sources suggests that proteins would be synthesized and stored under nutritionally rich conditions. Hence, the epimastigote proteins might function as the food supply for the trypomastigote stage.

Two experimental observations corroborate the idea of protein storage as a metabolic reserve within *T. cruzi*:

1 – Epimastigotes from LIT medium incubated in TAU for 2 hours and then labelled with 35S-methionine in Hanks' solution for 2 hours displayed a single protein profile. These pre-labelled parasites were incubated in TAUP medium (for time periods varying up to 96 hours) and the proteins were analysed by electrophoresis without additional labelling. The cells displayed a rather complex protein profile, similar to that obtained for metacyclic trypomastigotes; no net changes were observed in the amount of hot TCA precipitable material (Goldenberg et al., 1984b).

2 – Dm 28c metacyclic trypomastigotes display different profiles of *in vivo* labelled polypeptides depending upon their origin (Goldenberg et al., 1984a) (i.e., from TAUS medium, TAUP medium, Roitman chemically defined medium, A9 fibroblasts, LIT medium and triatomine urine). This indicates that *T. cruzi*, depending upon the environmental conditions, synthesizes proteins that might act as metabolic reserves.

Effect of fresh serum on gene expression: facts

The nutritional stress is not the only factor affecting mRNA translation in *T. cruzi*. We have observed that metabolic labelling of metacyclic trypomastigotes either derived from triatomines or induced *in vitro*, incubated with fresh guinea pig serum prior to 35S-methionine addition, results in a decrease in the rate of isotope incorporation into hot TCA precipitable material in comparison to control parasites incubated with heat inactivated (56 C, 30 min) serum. This result suggests a role for a serum heat labile component in the modulation of *T. cruzi* metacyclic trypomastigote protein synthesis. The analysis of the proteins synthesized under these two conditions shows that parasites incubated with fresh guinea pig serum stop synthesizing most of the proteins, mainly synthesizing the two surface antigens previously mentioned and of molecular weights 86,000 and 78,000 (Contreras, Morel & Goldenberg, 1985b).

Effect of fresh serum on gene expression: hypothesis – The hypothesis assumes that the fixation of the serum heat labile component to the surface of the parasite would polarize the flow of magnesium to the surface, diminishing the cytoplasmic concentration of the ion. This would provoke a reduction in the amount of functional polyribosomes in such a way that cytoplasmic mRNAs would compete for the initiation of protein synthesis. Hence the mRNAs that initiate better would be preferentially translated. Since initiation factors are not able to discriminate the mRNAs to be translated (Vicent et al., 1981), the choice of the initiating mRNAs would be dictated by the secondary structure of the messenger RNA 5'-noncoding region and the position of the initiator AUG codon in such structure (Pavlakis et al., 1980).

The nature of the serum heat labile component – As yet, the nature of the serum heat labile component is not known. We are presently investigating whether it could be a factor involved in the alternate pathway for complement activation, since components of this pathway are heat labile (Muller-Eberhard, 1975) and recent results showed that, although resistant to complement mediated lysis (Nogueira Bianco & Cohn, 1975), metacyclic trypomastigotes might fix complement (Kipnis, Krettli & Dias, 1984). It is worth mentioning that magnesium is an essential factor in the activation of the alternate pathway for complement (Muller-Eberhard, 1975).

Cytoplasmic control of *T. cruzi* gene expression

The mRNAs from Dm 28c epimastigotes and metacyclic trypomastigotes were characterized by their *in vitro* translation products in the nuclease treated rabbit reticulocyte lysate. The comparison of polyribosomal mRNAs from both differentiation stages has allowed the identification of stage specific mRNA sequences. When we analysed the post-polyribosomal mRNAs from both differentiation stages we found a less complex mRNA population, in equilibrium with the polyribosomal counterparts (Goldenberg et al., 1985). This result suggests that mRNAs from a particular differentiation stage are not stored as translationally repressed post-polyribosomal mRNAs (Vincent et al., 1981) in the other differentiation stage.

The comparison of *T. cruzi in vivo* and *in vitro* translation products shows several differences suggesting that post-translational processing of proteins is an important phenomena in this parasite. Indeed, we have confirmed this observation by immunoprecipitating *in vivo* and *in vitro* synthesized polypeptides with different antisera: the same antisera recognized polypeptide chains displaying different molecular weights depending upon whether they have been synthesized by living cells or rabbit reticulocyte lysate (Salles et al., in preparation).

Perspectives and concluding remarks

The hypothesis proposed in this work need to be tested further. We have recently constructed genomic and cDNA libraries from epimastigotes and metacyclic trypomastigotes. The mapping of stage specific genes within the genome could provide direct information on DNA rearrangement within the parasite. In addition, nucleotide sequence studies will provide useful data on gene structure and organization and whether the secondary structure of *T. cruzi* mRNAs could account for preferential translation of some mRNA sequences.

The development of studies on the genetics of *T. cruzi* will be certainly very useful for the understanding of the biological behavior of this complex unicellular eukaryote. Similarly, mutagenesis studies (Marchand & Boon, 1984) with this parasite will allow the selection of mutants for trypomastigote stage specific biological properties and metabolic pathways.

The TAUP medium developed in our laboratory will certainly be a useful tool in approaching several problems in *T. cruzi* research. The fact that high yields of *bona fide* metacyclic trypomastigotes are obtained in this medium, along with the observation that a single pattern of synthesized proteins accounts for the most relevant biological properties of the infective form of *T. cruzi*, facilitates the identification of the genes involved in the process of both infection and differentiation. This might be an important contribution to the therapeutics and immunoprophylaxis of Chagas' disease.

ACKNOWLEDGEMENTS:

We thank Drs. J. Ramalho Pinto and H. Momen for the critical reading of this manuscript and J.L. Aires Pereira and M.V. Soares Bastos for advise during its preparation.

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