Rev. Inst. Med. trop. São Paulo
25(2):55-61, *março-abril,* 1983

UDC 593.161.13
616.937.3-092.9

CHARACTERIZATION OF TRYPANOSOMA CRUZI METACYCLIC FORMS DIFFERENTIATED FROM EPIMASTIGOTES. A COMPARATIVE STUDY BETWEEN BOTH STAGES

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SUMMARY

Epimastigotes (Epi) of **Trypanosoma cruzi** grown in Grace culture medium supplemented with extracts from **Triatoma infestans** digestive tract differentiate into metacyclics (Mtc) as determined by optical microscopy as well as their higher infectivity for mice. In the present communication a comparative study to set up the actual degree of differentiation between Epi and the resultant Mtc was performed. As happens with Epi, Mtc were satisfactorily washed and resuspended in saline solution without protein addition. Mtc were undamaged when resuspended in normal mammalian sera (NMS) and specific antibodies (abs) induced caps on this stage. On the other hand, Epi were lysed by NMS and no caps were induced by abs, as expected. The lectin-parasite interaction was similar in both stages when Con A or SBA were assayed indicating the presence of α-D-glucose, α-D-mannose, and α-D-galactose. N-acetylglucosamine was also detected in both stages, but while Epi reacted by DA and DF with WGA, Mtc only reacted by DF. The resistance to lysis by NMS, the capability to form caps and the reactivity pattern with the lectins assayed, indicated that Mtc were closer to the slender bloodstream forms than to the original Epi. The immune response stimulated by Mtc was higher than the one induced by Epi as the results of DAT and MIF indicate. This higher immune response might be related to the capability of the Mtc to resist the mammalian complement lysis, leading to a greater degree of infection.

INTRODUCTION

Culture metacyclic trypomastigotes have been used to study cell-parasite interaction with **Trypanosoma cruzi** [18]. This form is usually obtained in liquid culture media at the end of the growth period reaching at most 30-35% [2,3,18]. More recently, efforts were made to develop special culture media to optimize differentiation of epimastigotes to metacyclics. Successful cultures are usually prepared with an insect cell culture medium support, enriched with insect hemolymph and/or insect (kissing bugs) tissue extracts [7,31,32]. A continuous tissue culture-embryo cell-line from **Triatoma infestans** has also been established [21]. ISOLA et al. have recently reported a high percentage of differentiation of epimastigote to metacyclic form induce by means of insect tissue extracts in a cell free culture as determined by optical microscopy as well as by the higher infectivity of these forms for mice [7].

In the present study we attempt to set up some differential features other than morpholo-

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gy between the epimastigotes and the resultant metacyclic forms differentiated **in vitro.**

MATERIALS AND METHODS

All the experiments were performed with the Tulahuén (Tul) **T. cruzi** strain. Epimastigotes (Epi) were obtained after 4 days growth in a biphasic culture medium and metacyclics (Mtc) from the Grace culture medium enriched with **Triatoma infestans** digestive tract homogenates (GMTIIH) after 6-10 days growth, as previously reported [7].

Parasites ($1x10^7$/ml) were washed in phosphate buffered saline pH 7.2 (PBS) or PBS 1% albumin (PBS-alb) to ascertain if Mtc need a protective protein incorporated in the wash media [9]. The number of Epi or Mtc was determined in a Neubauer type chamber after washing up to 8 times. Only motile parasites were considered as undamaged.

Washed parasites were resuspended at $1x10^7$/ml concentration in PBS containing 25% normal rabbit serum (NRS) or normal human serum (NHS) to evaluate complement activity [17]. Similar samples with PBS plus 25% heat inactivated (56°C for 30 minutes) serum were simultaneously assayed as controls. Samples were incubated for one hour at 37°C and the lysis percentage calculated in a Neubauer type chamber.

Immune sera reactivity with membrane antigenic receptors was determined with human sera obtained from patients with chronic **T. cruzi** infections (IHS). A pool with 1/128 specific indirect immunofluorescent antibody titer was used. Heat inactivated IHS and NHS were assayed in 1/64 and 1/8 dilutions, respectively. Two batches of samples of 200 μl serum dilutions were incubated with $1x10^7$ cells/ml for 30 minutes, one at 4°C and the other at 37°C, simultaneously, to evaluate cap induction. The serum excess was eliminated by washing with PBS-alb, 3 times, at 6.000 g for 10 minutes, in a cold room, after the incubation period. The sediment was resuspended up to the former concentration using the adequate dilutions and species of antigamma globulin conjugate fluorescein. The resultant suspension was then reincubated for another 30 minutes at 4°C. Fluorescence excess was eliminated by washing and the samples were observed in a Carl Zeiss fluorescence epiiluminator microscope using a 25x Planapo objective [6].

The presence of some lectin receptors was assayed by direct agglutination (DA) and direct fluorescence (DF) tests [8], Parasites, 25 μl of $2x10^8$/ml (DA) or $1x10^7$/ml (DF), were incubated in a microplate during one hour at 37°C, with 25 μl of a dilution ranging from 500 to 0.25 μg/ml of Concanavalin A (Con A), wheat germ agglutinin (WGA) or soy bean agglutinin (SBA) (Sigma Chemical Co., St. Louis, Mo). Lectins conjugated with fluorescein isothiocyanate were used in the DF tests (Pierce Chemical Co., Rockland II). The specificity of the reaction was determined by comparing the above samples with others containing the specific sugar inhibitor (α D-glucose, α-D-mannose, N-acetylglucosamine and α-D-galactose).

Humoral and cell mediated immune response was evaluated in groups of 25-day-old male Rockland mice by inoculation of Epi (range from 10^2 to 10^8) as well as Mtc (range from 10^2 to 10^5) by intraperitoneal route. Individual bleeding was performed 45 days postinoculation and serum was separated to evaluate specific antibodies by direct agglutination test (DAT) [30] (10 mice per group). After the DAT was performed the sera of each group were pooled to study their neutralizing activity according to the method already reported [4], injecting 10 mice per group and measuring the survival of the mice during 30 days.

To evaluate cell-mediated immune response mice were killed from day 40 to 50 postinoculation and macrophage migration inhibition test was performed for individual animals. The methodology reported by SCHMUÑIS et al. [27] for cell collection, test performance and the calculation of percentage migration was followed. One hundred micrograms of antigen [13] measured as protein concentration [12] was used to inhibit the peritoneal cell migration.

RESULTS

Epi as well as Mtc were satisfactorily washed either with PBS or PBS alb. No lysis was induced in any case even after washing 8 times.

Epi obtained from biphasic medium were lysed either by NRS or NHS. Neither serum

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damaged the Mtc obtained from GMTIIH while the Epi remaining in the sample were lysed like those obtained from the biphasic media. Heat inactivation inhibited the lytic activity. Results with NRS are shown in Table I. Although the Mtc were undamaged by normal mammalian sera, agglutination of parasites in groups containing 6-8 Mtc was seen.

T A B L E I

Epimastigotes (Epi) and Metacyclics (Mtc): their behaviour after incubation with normal rabbit serum

Culture media	Percentage of		Lysis percentage of Mtc		Lysis percentage of Epi	
	Mtc	Epi	INRS*	NRS**	INRS	NRS
GMTIIH	50	50	2	8	8	72
GMTIIH	95	5	0	2	16	48
Biphasic	1	99	ND***	ND***	12	84

* — heat inactivated normal rabbit serum
** — normal rabbit serum
*** — not done

Granular fluorescence on the whole Epi surface was observed when Epi were incubated with IHS either at 4 or 37°C. On the other hand, capping was induced on Mtc by IHS at 37°C but prevented when incubation was performed at 4°C.

When lectin-parasite interaction was assayed, Con-A and SBA were reactive by DA as well as DF either with Epi or Mtc. With SBA, Mtc showed the highest fluorescence coincident with the undulating membrane. When WGA was used Epi reacted by both techniques while Mtc were only reactive by DF. The reactivity was inhibited in all cases when the specific sugar inhibitors were incorporated to the reaction. The data are summarized in Table II.

T A B L E II

Lectin interaction with **T. cruzi** Epimastigotes (Epi) and Metacyclics (Mtc)

Lectin	Epi		Mtc	
	DA°	DF°°	DA	DF
Con-A	10 μg*	1 μg	60 μg	2 μg
WGA	40 μg	1 μg**	Neg***	125 μg
SBA	20 μg	1 μg	60 μg	1 μg

* — minimum amount of lectin needed to be reactive
** — in a few experiments DF with WGA was reactive with higher lectin concentration (about 50-60 μg)
*** — not reactive even with the 500 μg of the lectin tested
° — direct agglutination
°° — direct fluorescence

When parasites were inoculated, the immune response of mice injected with Mtc was higher than the response achieved by mice injected with Epi. As shown in Table III, $1x10^2$ Epi failed to elicit antibodies while the same dose of Mtc induced a serologic conversion in 60% of the animals. Otherwise, titers tended to be higher in all the groups inoculated with Mtc than in those inoculated with Epi (Table III). Neutralizing tests did not show significant differences among the groups. When the macrophage migration inhibition test was evaluated mice infected with Mtc showed a positive inhibition while those receiving Epi showed a wide dispersion in the individual results but most of them remained negative. The antigen seems to enhance the macrophage migration as indicated by the migration index of the controls (Fig. 1).

T A B L E III

Direct agglutination test (DAT) in mice infected with Epimastigotes (Epi) or Metacyclics (Mtc)

Dose	Epi		Mtc	
	reactive°	titer*	reactive	titer
10^8	9	5.8	ND	ND
10^6	10	5.4	ND	ND
10^5	ND**	ND	10	6.4
10^4	6	4.2	8	6.0
10^2	0	0.0	6	5.0

° — number of reactive samples over the 10 assayed per group
* — the titers are the average of the values of the reactive sera only, and are expressed as $\log_2$
** — not done

DISCUSSION

Various differential features and behaviours have been reported among epimastigote, amastigote and trypomastigote forms [1,6,8,9,10,11,15,19,22,25]. The real meaning of those differences is hardly clear. They may be determined by the environment or may represent physiological features belonging to each stage of the parasite.

It has been reported that Epi can be washed with any saline solution while the bloodstream form is damaged unless some protective proteins are added to the wash solution [9]. The present results show that, like Epi, Mtc differentiated in GMTIIH do not need any protective protein and can be suitably washed with PBS.

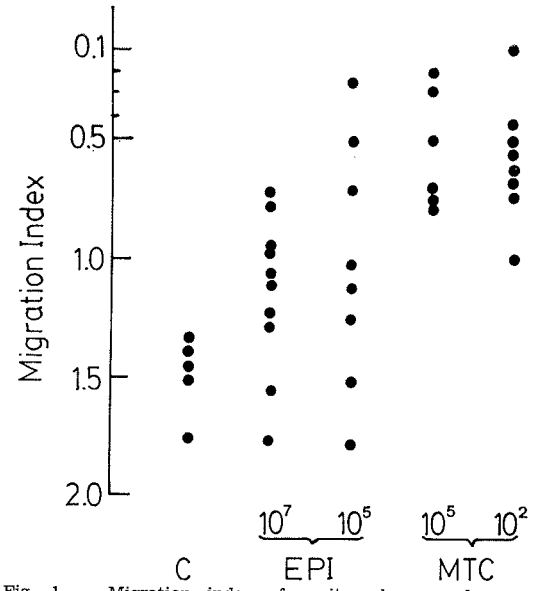

Fig. 1 — Migration index of peritoneal macrophages of mice inoculated with 10^7 or 10^6 epimastigotes or 10^5 or 10^2 metacyclics

Lysis of Epi by fresh mammalian sera has been reported [15,22]; the role of complement in this process has been studied and its activity utilized to purify Mtc differentiated in LIT culture medium [17]. The lower lytic activity induced by normal mammalian sera on Epi when differentiation is very high (see Table I) may be attributed to the higher error due to the low number of remaining Epi. Alternatevely, perhaps part of these Epi are undergoing differentiation, so that physiological changes could occur in spite of the apparently Epi-like morphology. The Mtc differentiated in GMTIIH proved resistant to the mammalian complement. The inability of the normal sera to damage the Mtc might be closely related to the higher infective capacity reported recently for these forms [7].

The higher degree of infectivity agrees with the better immune response stimulated in mice by Mtc as shown in the present studies. REIS et al. [23] have demonstrated that cell mediated immunity (CMI) response is induced by infection with any dose of 6 different strains tested. VATTUONE et al. [29] and SCHMUÑIS et al. [27] have communicated that CMI response (measured as macrophage migration inhibition test) appeared earlier in mice injected with trypomastigotes than in others injected with Epi. They also showed a close relationship between the amount of parasite-dose inoculated and the time the test becomes positive. Antibody response has also been reported as a sensitive test to evaluate successful infection [24,28]. In the present experiments DAT became positive in mice injected with any dose of Mtc while Epi induced less reactivity; these results are indicative of a different degree of successful infection depending on the parasite stage used. Anyway, the lack of NA+ in any of the groups assayed here is indicative that even the highest doses of parasites used, induced an infection unable to develop a NA+ up to 45 days. As the Tul strain had been proved to be able to stimulate neutralizing activity [4] we presume that the degree of infection is extremely low.

The caps induced by specific antibodies on the Mtc surface antigens indicate that the behaviour of this Mtc trypomastigote is similar to that of trypomastigotes obtained from blood or tissue cultures [6,25,26]. It had been postulated that trypomastigotes use this mechanism to avoid the host immune response; this idea is supported by the fact that caps are induced by antibodies **in vivo** [6]. On the other hand, antibodies are unable to induce caps on Epi [6,25]. The fact that trypomastigotes obtained from different sources showed the same potential capacity to form caps is indicative that the process is not dependent on the environment.

The interaction detected between Mtc surface and lectins establish the presence of α-D-mannose, α-D galactose, αD-glucose and N. acetylglucosamine, and their reactivity is similar to that demonstrated for the slender bloodstream population when DA and DF tests are used [5]. We consider our results to be in accordance with those communicated by PEREIRA et al. [19] although they had reported absence of N. acetylglucosamine in Mtc and in bloodstream forms of a slender strain by DA test. This apparent discrepancy might be dependent on the technique used by these authors. We detected this saccharide by DF, but never by DA. Similar DF and/or DA reactivity between bloodstream forms and WGA had been reported by ARAUJO et al. [1], GONZALEZ-CAPPA et al. [5] and KATZIN et al. [10].

Mtc and bloodstream forms are considered the most capable to achieve cell infection [14,17].

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Epi that are lysed by normal mammalian sera and are largely destroyed by macrophages [16], possess exposed N. acetylglucosamine, similar to Mtc and bloodstream forms, but agglutination by incubation with WGA was only positive with Epi. If the surface sugar is related to the infective capacity of the parasite stage is an interesting point which remains to be studied. A lower degree of infection of Vero cells had been reported for tissue culture trypomastigotes previously incubated with N. acetylglucosamine [20].

As far as the experimental protocols can be compared the behaviour of Mtc obtained from different culture media seems to be similar. But in the GMTIIH differentiation might be triggered more efficiently considering the larger number of Mtc obtained and the fact that the differentiation process is simultaneous with the duplication period [7]. In the present communication we have shown that Mtc differentiated in GMTIIH exhibit features different from the Epi stage, resembling bloodstream forms in some aspects. The surface saccharides detected and the ability of antibodies to induce capping are characteristics shared with the bloodstream forms, as well as the resistance to the complement lytic activity. These features may be responsible for their higher infective capacity which induce better immune response in mice as compared with that induced by Epi. On the other hand, Mtc like Epi, remain undamaged when washed and resuspended in saline solutions, being different in this sense from bloodstream forms.

RESUMEN

Caracterización de las formas metacíclicas del Trypanosoma cruzi **diferenciadas a partir de epimastigotas. Estudio comparativo entre ambos estados.**

Recientemente, se ha comunicado que cultivos preparados con un soporte adecuado para el crecimiento de células de insectos (Medio de Grace) enriquecidos con hemolinfa y/o extractos de **T. infestans** inducen una temprana diferenciación de epimastigotes (Epi) a tripomastigotes metacíclicos (Mtc) pudiendo obtenerse hasta un 90% de Mtc. Esta diferenciación ha sido evaluada por el aspecto morfológico presentado por los parásitos al microscopio óptico y por su mayor capacidad para infectar al ratón lactante o jóven. En el presente estudio se analizaron otras características distintas de las morfológicas con el fin de determinar el grado real de diferenciación alcanzado por Epi sembrados en un medio de cultivo enriquecido con extractos de intestino de **T. infestans** (GMTIIH). Cuando se evaluó el posible requerimiento de proteínas protectoras incorporadas al líquido de lavado se observó que los Mtc, al igual que los Epi, no las requieren. Los Mtc obtenidos en GMTIIH no fueron dañados por el agregado de suero normal de mamífero, a diferencia de los Epi que se lisaron como se esperaba. La resistencia a la lisis inducida por complemento de mamífero de los Mtc obtenidos tardíamente (al final del período de crecimiento) en medios líquidos y bifásicos ha sido comunicado por varios autores. Los resultados del estudio actual evidencian que la inmunorespuesta estimulada por los Mtc fue mayor, comparada con la inducida por los Epi, de acuerdo a los resultados obtenidos por las pruebas de aglutinación directa e inhibición de la migración de macrófagos; esta mejor estimulación de la inmunorespuesta podría estar relacionada con la infección más efectiva producida por los Mtc a consecuencia de su resistencia a ser lisado por sueros normales. Cuando se determinó la interacción lectina-parásito, ésta fue similar para ambos estadios frente a Con-A y SBA indicando la presencia de α-D-glucosa, α-D-manosa y α-D-galactosa. También se detectó N. acetilglucosamina en ambos estadios al enfrentarse los parásitos con WGA, pero mientras los Epi reaccionaron tanto por aglutinación como por fluorescencia directa, los Mtc sólo lo hicieron por fluorescencia directa, de manera similar a lo que se ha comunicado para los tripomastigotes circulantes en cepas a predominio de formas delgadas. Finalmente los Mtc fueron capaces de formar casquetes (caps) inducidos por acción de anticuerpos específicos. Esta capacidad así como el tipo de reactividad frente a las lectinas y la resistencia a la lisis por sueros normales de mamíferos indican que los Mtc diferenciados estarían más cerca de los tripomastigotes de cepas delgadas (slender) que de los Epi a partir de los cuales se originan.

ACKNOWLEDGMENT

This investigation received financial support from the UNDP/World Bank/Who (Spe-

cial Programme for Research and Training in Tropical Diseases) and from Secretaría de Estado de Ciencia y Tecnología from Argentina.
(*) Victor J. Katzin is a fellowshipholder of the research Carrier of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) from Argentina and (**) Stella M. González Cappa is a research member of the CONICET.

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Recebido para publicação em 9/11/1981.