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TRYPANOSOMA CRUZI: ACTIVITY OF IMMUNE SERA ON SURFACE ANTIGENS (*)

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SUMMARY

In vitro incubation of living **T. cruzi** epimastigotes with sera containing specific antibodies, either of mouse, rabbit or human origin, in the presence of anti-homologous species fluorescein isothiocyanate labelled immunoglobulins did not induce aggregation of surface antigens. Aggregation of surface antigens with cap formation was induced when blood stream or tissue culture trypomastigotes were incubated with an immune serum (IS) and subsequently with an anti-homologous species conjugated immunoglobulin. All the **T. cruzi** strains assayed in the present study reacted similarly. Trypomastigotes need to be metabolically active for cap formation since capping was inhibited by low temperature and sodium azide. It was induced when the parasites were incubated at 4.°C with IS and then at 37°C with the anti-homologous species conjugated immunoglobulins, or when the parasites were incubated at 37°C with IS and then either fixed before exposure to the conjugate or incubated at 4°C with the conjugate. This fact indicates that capping can be induced during incubation with the first ligand; therefore, capping might be a physiological phenomenon occurring **in vivo**. This hypothesis was corroborated when bloodstream parasites were processed at 4.°C directly with anti-mouse gamma globulin conjugate. Regeneration of antigenic determinants was also proved.

INTRODUCTION

Mammalian cells are capable of translocating and redistributing surface antigens which aggregated forming caps in the presence of antibodies or Concanavalin-A (RAFF & DE PETRIS [13]; NICOLSON [11]). This phenomenon has been reported for some protozoa: **Leishmania enrietti, Leishmania donovani, Toxoplasma gondii, T. cruzi, Entamoeba histolytica** (DOYLE et al.[4]; DOYLE et al.[5]; DZBENSKI & ZIELINSKA [6]; SCHIMUÑIS et al.[15]; TRISSE et al.[18]). It has been suggested that this reorganization of surface antigens may be the first step in either antigenic modulation, activation of the immune system or development of a host's tolerant state (RAFF & DE PETRIS [13]). If capped complexes are shed from the surface of the parasites these complexes may be responsible for some of the immunopathological lesions reported in several parasitoses (SZARFMAN et al [16]; TOSTA [17]).

In the present study we analysed the effect of specific antibodies, obtained from 3 different mammalian species, on the epimastigotes

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and trypomastigotes of several strains of **T. cruzi**, for their ability to induce mobilization and cap formation of surface antigens. Otherwise the possibility of capping **in vivo** was investigated.

MATERIAL AND METHODS

Parasites — Trypomastigotes of Tulahuén, Sayago, M65, 92, Esquilo, F and Y and epimastigotes of Y and Tulahuén strains of **Trypanosoma cruzi** were used. Bloodstream trypomastigotes were obtained from mice infected 7 days previously; in a few experiments trypomastigotes were used 9 and 13 days after infection; tissue culture trypomastigotes were harvested from the overlay of monolayer LLC-MK_2 cell cultures after 5 days infection. Epimastigotes were obtained from biphasic culture medium after 4 days of growth. Parasites purified by differential centrifugation were washed in phosphate buffered saline supplemented with 1% (v/w) bovine albumin F-V (PBS-alb) (KATZIN et al.[8]).

Immune sera — Human sera were obtained from patients in the chronic phase of Chagas' disease.

Mouse IS were prepared with the Tulahuén and Y strains as reported previously (GONZALEZ et al.[7]; KLOETZEL & DEANE[9]). Rabbit IS were obtained from animals which had received 15 weekly subcutaneous inoculations of trypomastigotes of the AWP **T. cruzi** strain (VATTUONE et al.[19]).

Normal sera (NS) from the same host species were used as controls.

Antiglobulin fluorescein labelled immunoglobulins — The conjugates purchased from Cappel Laboratories Inc. were anti-human immunoglobulin, anti-mouse serum, anti-mouse IgG and IgM, and anti-rabbit serum, prepared in goats and labelled with fluorescein isothiocyanate.

Fluorescein labelling of parasite membrane antigens — One million parasites were incubated with NS or IS, 1 vol. suspension of parasites/2 vol. sera, at 4.°C for 30 minutes. The sera had been previously heated at 56°C for 30 min. After incubation with IS and NS parasites were washed three times in cold PBS-albumin. Parallel samples of epimastigotes were incubated at 4°C and 28°C and trypomastigotes at 4°C and 37°C with anti-homologous species fluorescein isothiocyanate conjugates. In a few experiments trypomastigotes were processed with mouse IS or NS without heat inactivation.

To determine if capping was induced by the action of the first or the second ligand, trypomastigotes were incubated with the IS or NS at 37°C, washed in cold PBS-alb and part of the sample was further processed for 30 min. at 4°C and read immediately. The part not processed at 4°C was fixed with formalin (2% v/v in PBS) and dried in small drops on slides to be incubated at a later time with the proper conjugate at 37°C.

To find out if capping was induced **in vivo** some experiments were performed with blood trypomastigotes of the Tulahuén strain processed directly with anti-mouse IgG and IgM conjugate at 4°C immediately after purification and washing of the parasites; blood trypomastigotes were obtained from mice on days 7th, 9th and 13th after infection.

Preparations were read in a Carl Zeiss epi-illumination microscope with an epicondenser IV-FL and a halogen lamp of 12 V and 100 W.

Assay with a metabolic inhibitor — In some experiments replicate tubes containing trypomastigotes and antibodies, with or without sodium azide (Merck) 1 x 10^{-1} M, 5 x 10^{-2}M or 1 x 10^{-2}M solutions were processed.

RESULTS

When epimastigotes were sequentially incubated with IS and fluorescein conjugate, parasites showed a diffuse microgranular staining pattern when incubation was carried out either at 4°C or at 28°C. The staining pattern was similar when mouse rabbit or human sera were used.

When bloodstream or tissue culture trypomastigotes were employed the results obtained were similar for the **T. cruzi** strains assayed with the three species of sera used. When the sequential incubation with IS and fluorescein conjugate was carried out at 4°C, parasites showed the same diffuse microgranular staining pattern as did the epimastigotes. When incubation of trypomastigotes-IS was performed at 4°C and subsequently at 37°C with the conjugate, the diffuse microgranular staining pattern

changed to patch and cap formation (Table I and Plate 1). At this temperature after 30 minutes, 20-30% parasites appeared unstained and 30-50% parasites showed caps. Caps were present either at the anterior or posterior pole of the parasite. Some trypomastigotes showed 3 areas of aggregation, two at the poles and the third in another area. The remaining 20-40% of the parasites showed a microgranular pattern similar to that observed when the process was carried out at 4°C. Capping was similar regardless if the IS used was fresh or heat inactivated.

When trypomastigotes were exposed to IS at 37°C and the rest of the procedure was carried out at low temperature capping was also induced. Samples fixed with formalin after their incubation with IS at 37°C, when processed on slides as in standard IFI testes, also showed parasites with caps. Assays carried out with 10^{-1}M sodium azide destroyed the parasites; when 5 x 10^{-2}M sodium azide was used 60% of the parasites appeared non-motile and showed a linear fluorescent pattern similar to the standard IFI tests carried out with antigens prepared with dead parasites. When sodium azide was used at 1 x 10^{-2}M trypomastigotes incubated sequentially at 4°C and 37°C with IS and fluorescein conjugate were alive, with good motility and diffuse microgranular staining pattern (Table I).

TABLE I

Capping induced by antibodies on trypomastigotes and inhibition of this phenomenon

Stage 1*	State of the parasites after stage 1	Stage 2	Reactivity type
IS + T (4°C) **	Unfixed		Microgranular
IS + T (37°C) **	Unfixed	Anti-Ig•	Capping
IS + T + SA 10^{-2} M (37°C)	Unfixed	at 4°C for	Microgranular
IS + T + SA 5.10^{-2} M (37°C)	Unfixed	30 min	Lineal••
IS + T + SA 10^{-1} M (37°C)	Unfixed		Destruction
IS + T (37°C)	Fixed		Capping

IS: Immune sera T: Trypomastigotes SA: Sodium azide
*: 30 min. of incubation
**: When these conditions were assayed with normal serum the parasites were unstained
.: Conjugated with fluorescein isothiocyanate
..: Similar to the stain obtained when dead parasites are used in the performance of the normal diagnostic test.

In an attempt to determine if caps persisted once formed, as well as if antigens reappeared on the parasites surface, trypomastigotes were sequentially incubated at 37°C with IS and conjugate (Table II). After 30 min. of incubation with the conjugate 60% of the parasites showed caps, 35% diffuse microgranular fluorescence and 5% were unstained. After 120 minutes parasites with caps were reduced to 32% and negative forms went up to 35%, the number of parasites with a diffuse microgranular fluorescent pattern remaining approximately constant (33%). When parasites were washed and re-exposed to the IS and conjugate at 4°C, 80% showed the diffuse granular pattern of fluorescence, 10% retained caps and 10% were unstained (Table II).

When bloodstream parasites were incubated with anti-mouse IgG, those obtained on 9th

TABLE II

Reappearance of surface antigens of trypomastigotes after cap formation induced by immune sera

Time of observation	% of parasites with:		
	capping	granular	unstained
30 min. (37°C)	60	35	5
120 min. (37°C)	32	33	36
Re-exposed (*)	10	80	10

(*) After 120 min. part of the parasites were re-exposed to the immune sera and the conjugate (4°C)

and 13th days after infection showed positive reaction indicating that IgG antibodies were adhered to their membrane **in vivo**; 10% of these parasites possessed continuous granular fluorescence pattern, 75% of them showed caps and the rest were negative. When anti-mouse IgM was used no antibodies were detected on the surface of those trypomastigotes. Parasites obtained on day 7th were negative either with anti-IgG and anti-IgM conjugates (Table III).

TABLE III

In vivo distribution of immunoglobulins on the surface of trypomastigotes

Ig	Parasites obtained on day		
	7th	9th	13th
IgM	Not reactive	Not reactive	Not reactive
IgG	Not reactive	Caps (*) (65%)	Caps (*) (75%)

(*) Few parasites with microgranular and continuous fluorescence pattern were seen

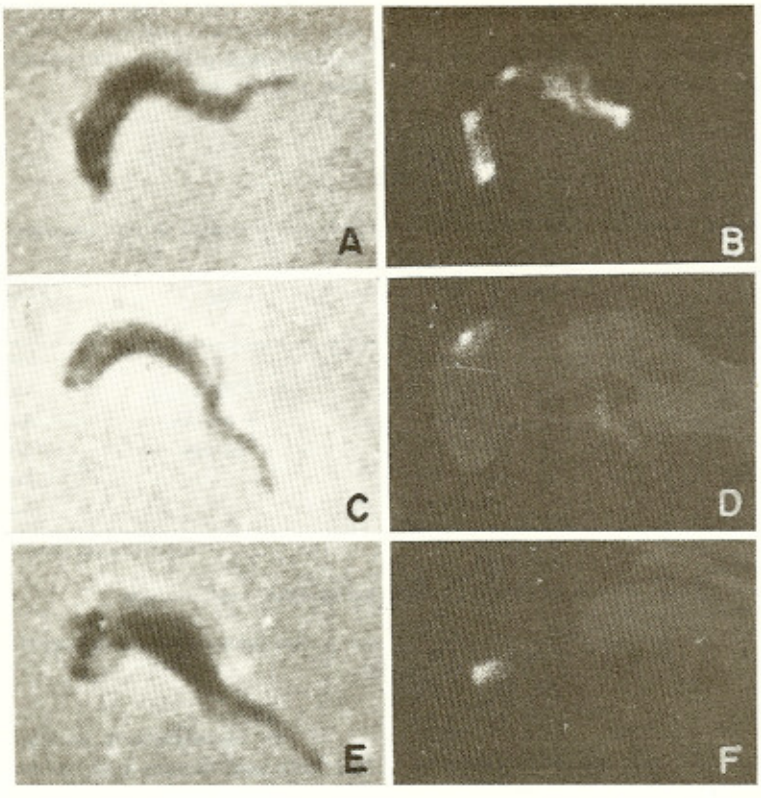

Plate 1 — Capping induced by antibodies on trypomastigotes: parasites observed after incubation at 4°C followed by incubation with isothyocyanate conjugated serum at 37°C. A, C and E — Blood trypomastigotes seen under phase contrast. B — Patching; the same parasite as in A; seen with dark field. D and F — caps; the same parasites as in C and E respectively, seen with dark field.

DISCUSSION

Capping of **T. cruzi** Y strain bloodstream forms, sensitized with specific antibodies, and sensitized trypomastigotes undergoing differentiation in LIT culture medium, were reported by other Authors (SCHMUÑIS et al.[15]; KLOETZEL & DEANE[9]).

Sera of the 3 species of the **T. cruzi** infected hosts tested, were found to contain antibodies which reacted with surface antigens of epimastigotes and trypomastigotes but which induced capping only in trypomastigotes. Caps were induced either in blood or tissue culture trypomastigotes and all the strains assayed were found to be active. Therefore, capping

seems to be a capability of the trypomastigotes form.

The inhibition of "caps" by sodium azide or by low temperature indicates that the parasites need to be metabolically active to perform capping. The percentage of capping reported in this experiment is similar to that reported for other parasites (DOYLE et al.[4]).

Capping induced by antibodies has been reported for intracellular protozoa such as **L. enrietti, L. donovani** and **T. gondii** (DOYLE et al.[4]; DOYLE et al.[5]; DZBENSKY & ZIELINSKA[6]), indicating that they possess defensive mechanisms against antibodies that may adhere to their membrane antigens. Even when these parasites are protected from the deleterious action of antibodies due to their **in vivo** intracellular location, specific immune sera possess activity against them **in vitro.** Not only capping but inhibitory action with regard to the growth of leishmanias and opsonic activity against **T. gondii** have been reported (ADLER[1]; ARAUJO[2]).

DOYLE et al.[4,5], have reported capping for **L. enrietti** and **L. donovani** in the intracellular amastigote stage in mammals and the promastigote stage in biphasic cultures. We were unable to detect any antigenic surface reorganization in culture forms with **T. cruzi.** Epimastigotes are usually lysed by normal mammalian sera through the alternate complement pathway (RUBIO[14]; NOGUEIRA et al.[12]). Specific complement dependent immune lysis has been also reported for this stage (YANOVSKY et al.[20]; ANZIANO et al.[3]). Even though specific immune lysis of epimastigotes was demonstrated, the fact that these forms are readily destroyed by normal mammalian sera is an indication of the high lability of epimastigotes; because of this lability defensive mechanisms may be absent.

KLOETZEL & DEANE[9] and KRETTLI & NUSSENZWEIG[10] have reported that parasites recovered from the mouse blood have antibodies adhered to their surface. KRETTLI & NUSSENZWEIG[10] reported also that human complement, when added to the parasites, induced lysis of the Y strain while CL strain was not affected, even when both possessed antibodies on their surface; this might be an indication of different organization between the surface antigens of both strains.

The evidence that the capping was induced **in vitro** by the first ligand was indicative that similar activity might be accomplished **in vivo** and this was demonstrated when circulating parasites were incubated at low temperature with anti-mouse gamma globulin conjugate.

The capping **in vivo** might represent one of the mechanisms used by the blood stage to avoid destruction by circulating antibodies. This mechanism might also be an explanation for the persistence of low parasitemias in hosts with chronic infections. If the immunocomplexes are being shed, this phenomenon might be responsible, at least in part, for the immunopathology already described.

RESUMO

Trypanosoma cruzi: ação de soros imunes sobre antígenos de superfície

A incubação **in vitro** de epimastigotas vivos de **T. cruzi** com soros de camundongo, coelho ou humano contendo anticorpos específicos, seguida de imuneglobulinas anti espécie homóloga, conjugadas com isotiocianato de fluoresceína, não induziu a agregação de antígenos de superfície. Agregação de antígenos de superfície com formação de "caps" foi induzida em tripomastigotas sanguícolas ou de cultura de tecidos incubados com soro imune (IS) e subsequentemente com imuneglobulina conjugada, anti espécie homóloga. Todas as cepas de **T. cruzi** testadas neste estudo reagiram de maneira semelhante. Os tripomastigotas precisam estar com metabolismo ativo para que se formem "caps" pois o fenômeno foi inibido em temperaturas baixas e na presença de azida sódica. Era induzido quando se incubavam os parasitas a 4°C em IS, seguido de incubação a 37°C com as imuneglobulinas conjugadas anti espécie homóloga, ou quando se incubavam os parasitas com IS a 37°C, sendo fixados antes de expostos ao conjugado, ou incubados com a mesma a 4°C. Este fato indica que o "capping" pode ser induzido durante a incubação com o primeiro ligante; portanto o "capping" poderia ser um fenômeno fisiológico que ocorre **in vivo.** Esta hipótese foi corrobada ao se tratarem parasitas sanguícolas diretamente com o conjugado anti gamaglobulina de camundongo a 4°C. Também foi provada a regeneração de determinantes antigênicos.

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