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F(ab')$_2$ antibody fragments against *Trypanosoma cruzi* calreticulin inhibit its interaction with the first component of human complement

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ABSTRACT

Trypanosoma cruzi calreticulin (TcCRT), described in our laboratory, retains several important functional features from its vertebrate homologues. We have shown that recombinant TcCRT inhibits the human complement system when it binds to the collagenous portion of C1q. The generation of classical pathway convertases and membrane attack complexes is thus strongly inhibited. In most *T. cruzi*-infected individuals, TcCRT is immunogenic and mediates the generation of specific antibodies. By reverting the C1q / TcCRT interaction, a parasite immune evasion strategy, these antibodies contribute to the host / parasite equilibrium. In an *in vitro* correlate of this situation, we show that the C1q / TcCRT interaction is inhibited by F(ab')$_2$ polyclonal anti-TcCRT IgG fragments. It is therefore feasible that in infected humans anti-TcCRT antibodies participate in reverting an important parasite strategy aimed at inhibiting the classical complement pathway. Thus, membrane-bound TcCRT interacts with the collagenous portion C1q, and this C1q is recognized by the CD91-bound host cell CRT, thus facilitating parasite internalization. Based on our *in vitro* results, it could be proposed that the *in vivo* interaction between TcCRT and vertebrate C1q could be inhibited by F(ab')$_2$ fragments anti-**r**TcCRT or against its S functional domain, thus interfering with the internalization process.

Key terms: calreticulin, C1q, F(ab')$_2$ antibody fragments, *Trypanosoma cruzi*

INTRODUCTION

Trypanosoma cruzi (*T. cruzi)* is a protozoan parasite that causes Chagas' disease, a major public health problem in Latin America (Fernández, 2002). In 1991, we identified Tc45, a *T. cruzi* immunogenic molecule (Ramos et al., 1991). Cloning and sequencing of the Tc45 coding gene revealed important degrees of identity with calreticulin from several vertebrate and invertebrate species. Thus, we proposed that Tc45 is calreticulin from *T. cruzi*, an electrophoretic dimorphic protein with the coding genes located in variable numbers of chromosomes (Aguillón et al., 1995; Aguillón et al., 2000). TcCRT exhibits interesting anti-angiogenic properties and structural and functional homologies with HuCRT, such as being a pleiotropic chaperone molecule (Ferreira et al., 2004a; Molina et al., 2005; Ferreira et al, 2005). Moreover, TcCRT inhibits *in vitro* the classical pathway of the human complement system (Ferreira et al., 2003; Ferreira et al., 2004b), with possible immune evasion consequences. Most likely, this effect occurs *in vivo*, given the presence of TcCRT on the parasite surface (Ferreira et al., 2004b; Souto-Padron et al., 2004).

In most, if not all, *T. cruzi*-infected individuals, TcCRT is immunogenic and mediates the generation of variable serum levels of specific antibodies (Molina et al., 2003). Most likely, these antibodies play a

role in the modulation of the C1q / TcCRT interaction, possibly reverting, at least partly, this immune evasion parasite strategy. Within this context, we have developed an *in vitro* correlate of this situation, showing that it is possible to specifically inhibit the TcCRT / human C1q interaction with heterologous polyclonal IgG anti-TcCRT.

MATERIALS AND METHODS

Production of polyclonal antisera to purified rTcCRT and its S functional domain

Two 6-month-old New Zealand White female rabbits (Institute of Public Health, Chile) were bled from the central ear artery, as a source of pre-immune sera. Four days later, one animal was immunized subcutaneously with 150 μg of **r**TcCRT, while the other received the same dose of the S functional domain (TcS) (aa 159-281) (Ferreira et al., 2004b). Both inocula contained Complete Freund's Adjuvant (CFA) (Sigma, St. Louis, MO, USA). On two subsequent occasions, 7 days apart, the rabbits were injected with 150 μg of antigens in Incomplete Freund's Adjuvant (Sigma). The animals were bled once a week, starting 7 days after the third immunization. A third rabbit was inoculated with CFA, as a negative control. Reactivity of the two experimental antisera, their respective pre-immune sera and the serum from the rabbit immunized with CFA was evaluated by enzyme-linked immunosorbent assay (ELISA) and immuno-Western blotting (IWB) against **r**TcCRT, TcS and TcR (aa 136-281) domains (Ferreira et al., 2004b) and whole epimastigote extract. All immunizations and bleeding procedures were performed under veterinary supervision and the animals were kept under standard internationally accepted conditions (National Research Council, 1996).

Purification of nTcCRT

Ammonium sulphate (50% of saturation, at 4°C) precipitated IgGs anti-TcCRT were further purified in a Sepharose-Protein G column (Pharmacia Fine Chemicals, Uppsala, Sweden). The biological activity and integrity of the purified immunoglobulins were determined by ELISA and IWB, respectively. These immunoglobulins were coupled to a BrCN-activated Sepharose column (Sigma), using standard procedures (Hermanson et al., 1992; Amersham Pharmacia Biotech AB, 2001). **n**TcCRT was purified from 3 ml of *T. cruzi* epimastigote axenic culture, Tulahuén strain, collected at the exponential growth phase (donated by Dr. Y. Repetto, ICBM, Medicine School, Universidad de Chile). A parasite extract prepared by standard procedures (Aguillón et al., 1995) was loaded onto the Sepharose-anti TcCRT Ig column. The mobile and stationary phases were rotated overnight, at 4°C. Then, the column was washed exhaustively with PBS and eluted with 10 ml glycine-HCl 0.1M, pH 2.8 and neutralized with Tris-HCl 1M, pH 8.6. The presence of **n**TcCRT was determined by IWB and Coomassie Blue R-250 staining (Merck, Darmstadt, Germany) (Aguilar et al., 2003).

Generation of rabbit IgG F(ab')$_2$ fragments anti-rTcCRT and TcS domain

Performed as described previously (Coligan, 1991). Briefly, 5.1 mg of Sepharose protein G-purified rabbit IgG, anti-TcS and anti-**r**TcCRT, were dialyzed against acetate buffer, pH 4. Then, pepsin (0.1 mg/ml), dissolved in the same buffer, was added at a 1: 20 (w/w) enzyme: antibody ratio. After incubating for 12 hrs at 37°C, the reaction was stopped by raising the pH to 8 upon addition of 50 μl/ml of 2M Tris base. Undigested Fc fragments were removed with Agarose-Protein G. A similar procedure was used to generate F(ab')$_2$ fragments from a rabbit injected with CFA. After dialysis, the purity and biological activity of the obtained F(ab')$_2$ fragments were confirmed by standard SDS-PAGE and ELISA.

Interaction of nTcCRT and TcS with human C1q

ELISAs were performed using Nunc Maxisorb plates (Fisher Scientific, Pittsburgh, PA, USA) coated with variable

concentrations of **n**TcCRT and TcS, diluted in PBS. As controls, wells received buffer alone or with 2.5% w/v BSA. Nonspecific binding sites were blocked with 2.5% w/v BSA in PBS. Each step was followed by washings with PBS/0.05% Tween 20. Then, increasing amounts of pure human C1q (Sigma) were added to a final volume of 100 µl PBS / 1% w/v BSA/0.05% Tween 20. Bound C1q was detected with rabbit anti-human C1q antibodies (DAKO, Carpinteria, CA, USA), followed by HRP-conjugated and affinity purified, goat IgG anti-rabbit Igs (DAKO). The reaction was read at 405 nm.

Modulation of the TcCRT / human C1q interaction by anti-rTcCRT and TcS immunoglobulin F(ab')$_2$ fragments

Briefly, PVC plates were sensitized overnight, at 4°C, with 4 mg/ml of TcS or **r**TcCRT in PBS. After each step, the plates were washed exhaustively. The remaining sites in the solid phase were blocked with an irrelevant protein. The plates were incubated 1 hr at 37°C with 100 µl of three different concentrations of F(ab')$_2$ fragments (50, 5 and 0.5 µg/ml) and a constant 1 µg/ml pure human C1q concentration (Sigma). The plates were then incubated with goat anti-human C1q antibodies (Sigma), followed by rabbit antibodies, coupled to peroxidase, against goat immunoglobulins (Calbiochem, San Diego, CA, USA). The rest of the procedure was similar to that described in the previous paragraph.

RESULTS

The presence of antibodies against **r**TcCRT, TcS, TcR and **n**TcCRT (purified or in the context of a whole epimastigote extract) was verified by IWB developed with the rabbit immune sera, as shown in Fig. 1. An anti-TcCRT monoclonal antibody (Aguillón et al., 1997) was used as a positive control (Fig. 1a). In general, the recombinant proteins (Fig. 1a-c, **r**TcCRT, Dom S and Dom R) are more susceptible to degradation than the native counterpart (Fig. 1a-c, Epimastigote Extract). The pre-immune sera did not react with the recombinant protein or its domains, nor with the native counterpart (results not shown). The immune rabbit serum and the monoclonal anti-TcCRT antibody recognize the same banding pattern in the recombinant and native proteins, except for a 73 kDa band which is recognized only by the former (Fig. 1b-c). In Fig 1d, lanes 2-4 from left to right, show affinity purified **n**TcCRT, respectably detected with the E2G7 monoclonal antibody, the polyclonal serum and also with Coomassie blue.

The generation of immunoglobulin fragments involves several stringent steps. It was important, therefore, to demonstrate that our F(ab')$_2$ fragments were biologically active (i.e., they recognize TcCRT or its domains). The results are summarized in Fig. 2. Thus, the activity of the serum that served as source of immunoglobulins is shown in Fig. 2a. Fig. 2b shows a reducing, Coomassie blue-stained, SDS-PAGE of the fragments and the original immunoglobulins. The biological activity of the F(ab')$_2$ fragments is shown in Fig. 2c.

Binding assays indicated that both **r**TcCRT and immunoaffinity purified **n**TcCRT, tested at equimolar concentrations, interact with C1q, in a concentration dependent, saturable and specific manner (results not shown). As expected, this property is shared by TcS, the functional TcCRT domain (Fig. 3a). This last assay also was useful for determining a suboptimal TcS concentration to be used in the assays intended assess the modulatory effects of the F(ab')$_2$ fragments (Fig. 3b). These experiments were performed with recombinant proteins, given their functional homology with the native counterpart. ELISA plates were sensitized with a constant concentration of **r**TcCRT or TcS. In both cases, variable concentrations of anti-**r**TcCRT, anti-TcS and control F(ab')$_2$ fragments were added, followed by a fixed pure human C1q concentration. Different degrees of reversion of the interactions between C1q and the recombinant proteins were observed. Thus, about 50% reversion of the interactions of C1q with **r**TcCRT or with TcS was mediated by only 5 µg/ml of total anti-TcS F(ab')$_2$ fragments. A maximum inhibition of 70-75% is reached with a logarithmic increase in the concentrations of these fragments.
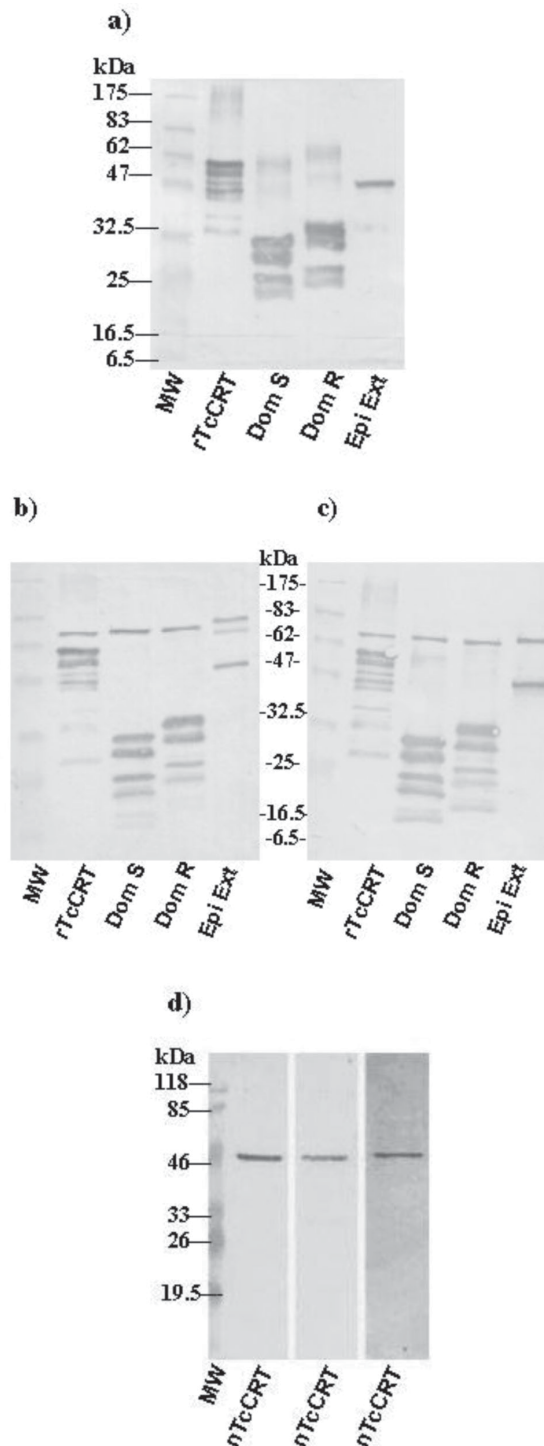
Fig. 1. Immunoenzymatic detection by immuno-Western blotting of rabbit antibodies against nTcCRT (present in a parasite extract), rTcCRT and its TcS, TcR domains. Source of second antibody: a) monoclonal antibody E2G7; b) rabbit immune serum anti-**r**TcCRT; c) rabbit immune serum anti TcS. In Fig. 1d) the second antibodies are (from left to right): E2G7 and rabbit serum anti **r**TcCRT. The last lane corresponds to a Coomassie blue-stained control. (As usual, the recombinant proteins, unlike the native counterpart, show a tendency to degradation and oligomerization).
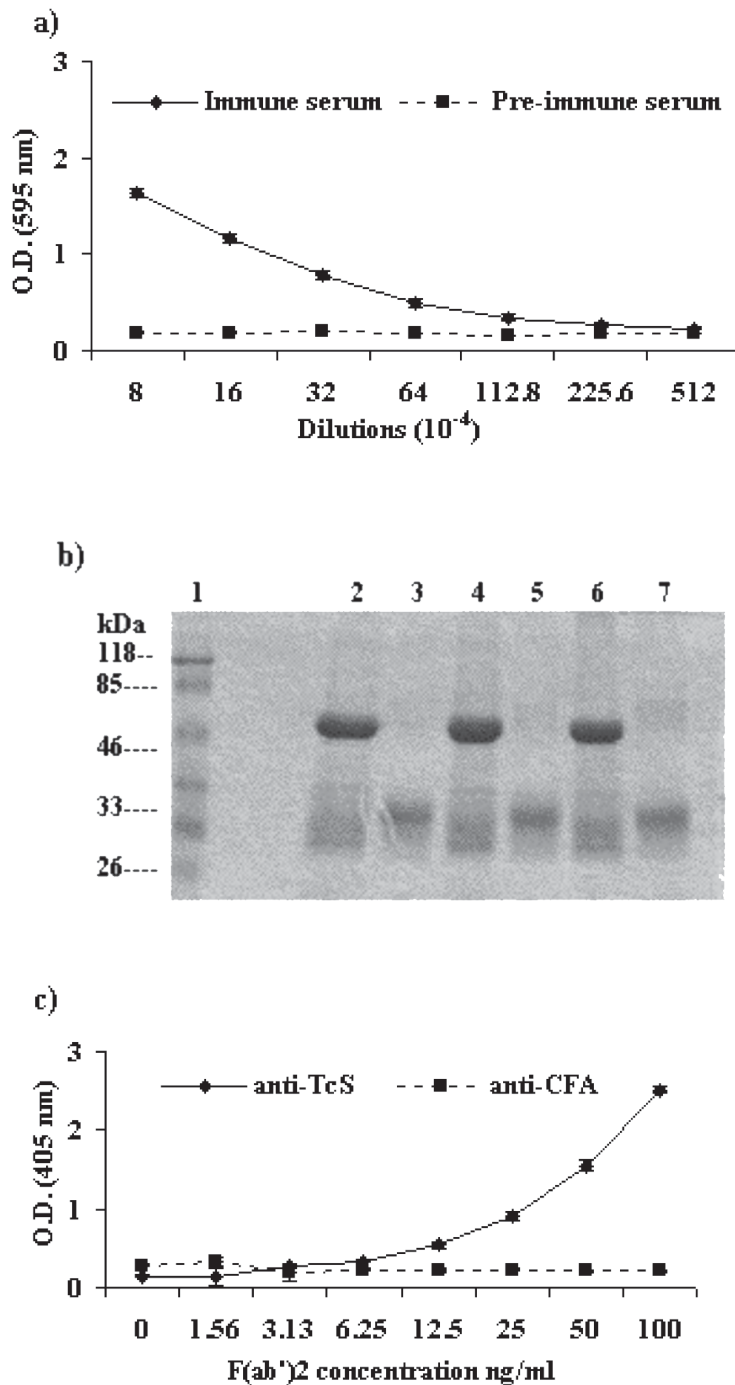
Fig. 2. Generation of functional anti-TcCRT F(ab')$_2$ fragments: a) Titration of rabbit antiserum against recombinant TcS by ELISA**.** Antigen: TcS. Antibody source: Rabbit immune and pre-immune sera. Each value corresponds to the mean of three measurements per dilution. (Standard deviations are too small to be visible in this figure). b) Coomassie blue-stained SDS-PAGE of whole IgG and F(ab')$_2$ fragments (anti-**r**TcCRT, anti-TcS and anti-CFA). 1: Pre-stained MW standard. Lanes 2, 4, 6: whole IgG (anti-**r**TcCRT, anti-TcS and anti-CFA, respectively) and lanes 3, 5, 7 show their corresponding F(ab')$_2$ fragments. c) Biological activity of the F(ab')$_2$ fragments. Assay: ELISA. Antigen: TcS. Each value corresponds to the mean of three measurements per dilution. Vertical bars indicate standard deviations.
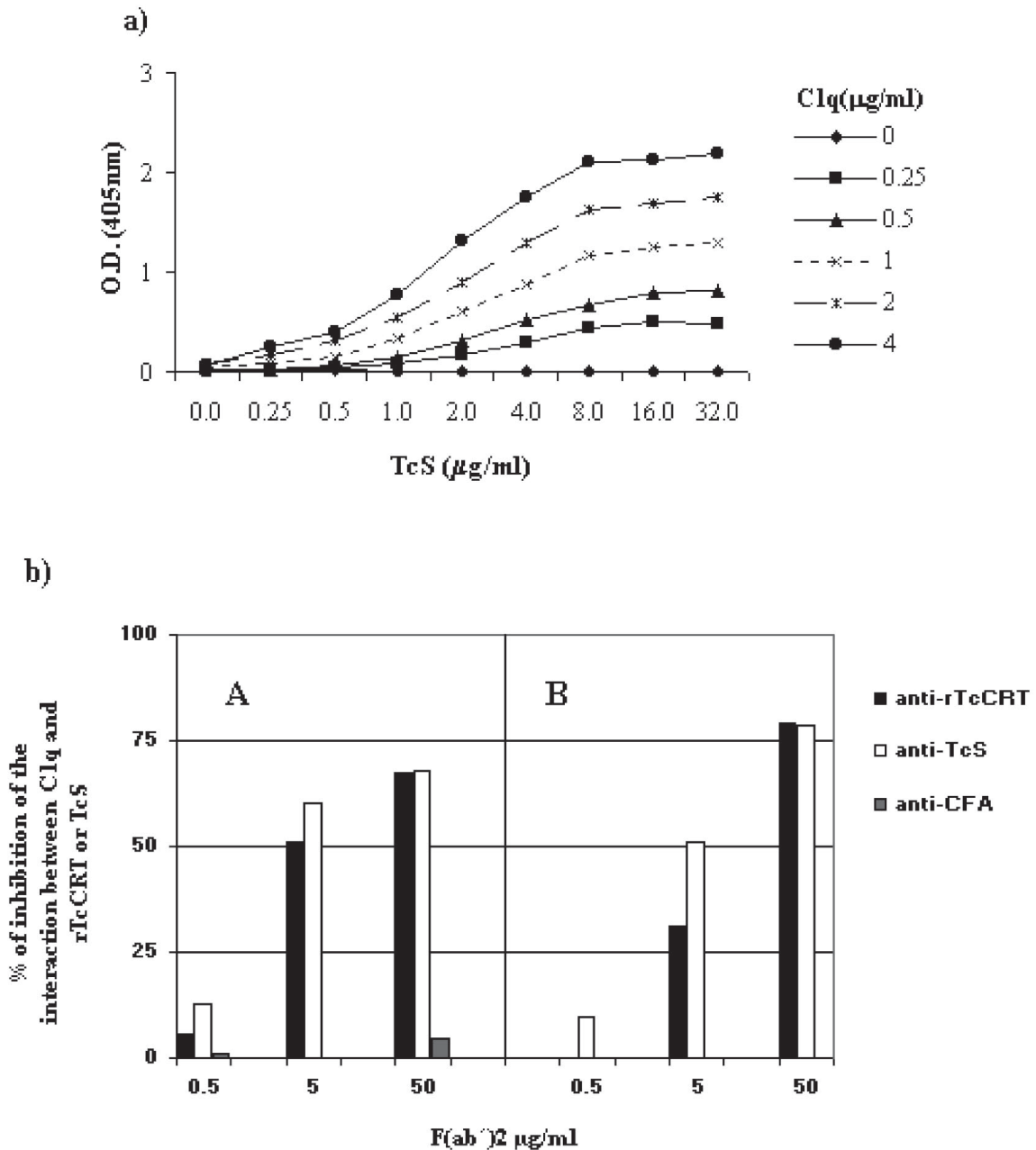
a)

b)

Fig. 3. Interaction of TcCRT with human C1q and modulation by specific antibodies.
a) Binding of TcS to human C1q. Assay: ELISA. Each curve represents the interaction between variable concentrations of both C1q and TcS. (Standard deviations are too small to be visible in this figure). b) Modulation of the interaction between human C1q and **r**TcCRT or TcS, by the corresponding $F(ab')_2$ fragments: the microtitration wells were sensitized with **r**TcCRT (A) or with TcS (B). Three different concentrations of $F(ab')_2$ fragments anti-**r**TcCRT and anti-TcS were tested. $F(ab')_2$ fragments prepared with sera from animals injected with Complete Freund's Adjuvant were used as negative controls. The experiments were performed twice with similar results.

DISCUSSION

Recent data from our laboratory indicate that *T. cruzi* calreticulin (TcCRT) shares a series of important functions with its vertebrate homologue. Thus, recombinant TcCRT (**r**TcCRT), or derived functional domains (TcS and TcR), specifically bind to the collagenous tails of the initial human complement component C1q, with important inhibition of the classical pathway (Ferreira et al., 2004b). It is possible, therefore, that *T. cruzi* utilizes TcCRT to modulate, in its benefit, this important immune response arm. Thus, although both epimastigotes and trypomastigotes display TcCRT on their surfaces, only in the latter is the S domain exposed (unpublished results). Perhaps, this differential TcCRT expression could partly explain the fact that unlike trypomastigotes, non-infective axenic culture-derived epimastigotes are highly susceptible to complement-mediated lysis.

On the other hand, since most *T. cruzi*-infected humans generate variable serum levels of specific antibodies against TcCRT (Aguillón et al., 1997; Marcelain et al., 2000), it could be proposed that the TcCRT / C1q interaction is inhibited by these antibodies, with benefits for the host. Within this context, in an *in vitro* correlate of this situation, we show here that the TcCRT / human C1q interaction is inhibited specifically by polyclonal IgG anti the whole recombinant molecule and its TcS functional domain. It was necessary to generate bivalent $F(ab')_2$ fragments from these antibodies in order to eliminate the possibility of complement activation via the Fc portions of the relevant immunoglobulins.

The presence of antibodies against **r**TcCRT, TcS, TcR and affinity-purified or epimastigote extract-derived **n**TcCRT was verified by IWB developed with the rabbit immune sera (Fig. 1). In general, the recombinant proteins are more susceptible to degradation than the native counterpart. The immune rabbit polyclonal serum recognizes a 73 kDa band, while the monoclonal antibody fails to do so. This band could correspond to dimers of the recombinant or native molecules, where the epitope recognized by the monoclonal antibody is not exposed.

Since the generation of immunoglobulin fragments involves several stringent steps, the biological activity of the $F(ab')_2$ fragments (i.e., capacity to recognize TcCRT or its domains) was verified, as shown in Fig. 2. Since the IgG concentration in whole serum is about 12 mg/ml, and the titer of the product used was 1/640,000 (Fig. 2a), the 12.5 ng/ml of fragments necessary to generate a similar activity are approximately within the same order of magnitude. It is generally accepted that in a polyclonal hyperimmune antiserum, no more than 5% of the total immunoglobulins are specific for the relevant antigen.

The anti–TcCRT $F(ab')_2$ fragments inhibit the interaction between the parasite molecule and C1q. Variable degrees of reversion of the interactions between the parasite molecule and the complement component were observed, indicating that the immunoglobulin fragments effectively blocked the binding. Thus, about 50% reversion of the C1q / **r**TcCRT or TcS interactions were mediated by only 5 μg/ml of total anti-TcS $F(ab')_2$ fragments. These results are compatible with the notion that the capacity of TcCRT to bind C1q and, as a consequence, to inhibit the classical complement pathway can be inhibited by $F(ab')_2$ fragments against TcS or **r**TcCRT. In other words, the anti-TcCRT antibodies generated during the trypanosomic infection may play an important role in the equilibrium frequently generated between the host and the parasite.

Perhaps, as a biotechnologic implication of these findings, $F(ab')_2$ fragments could be experimentally used *in vivo* or *in vitro* in order to interfere with the parasite infectivity. In Fig. 4, a schematic representation of a possible role of the C1q / TcCRT interaction in parasite infectivity is proposed. The mechanisms by which $F(ab')_2$ fragments could intervene these interactions are also depicted. Thus, parasite membrane-bound TcCRT specifically interacts with C1q (Ferreira et al., 2004a) (A). C1q, on the parasite membrane, is recognized by the CD91-bound host cell CRT (Basu et al., 2001; Gardai et al., 2003) (B), thus
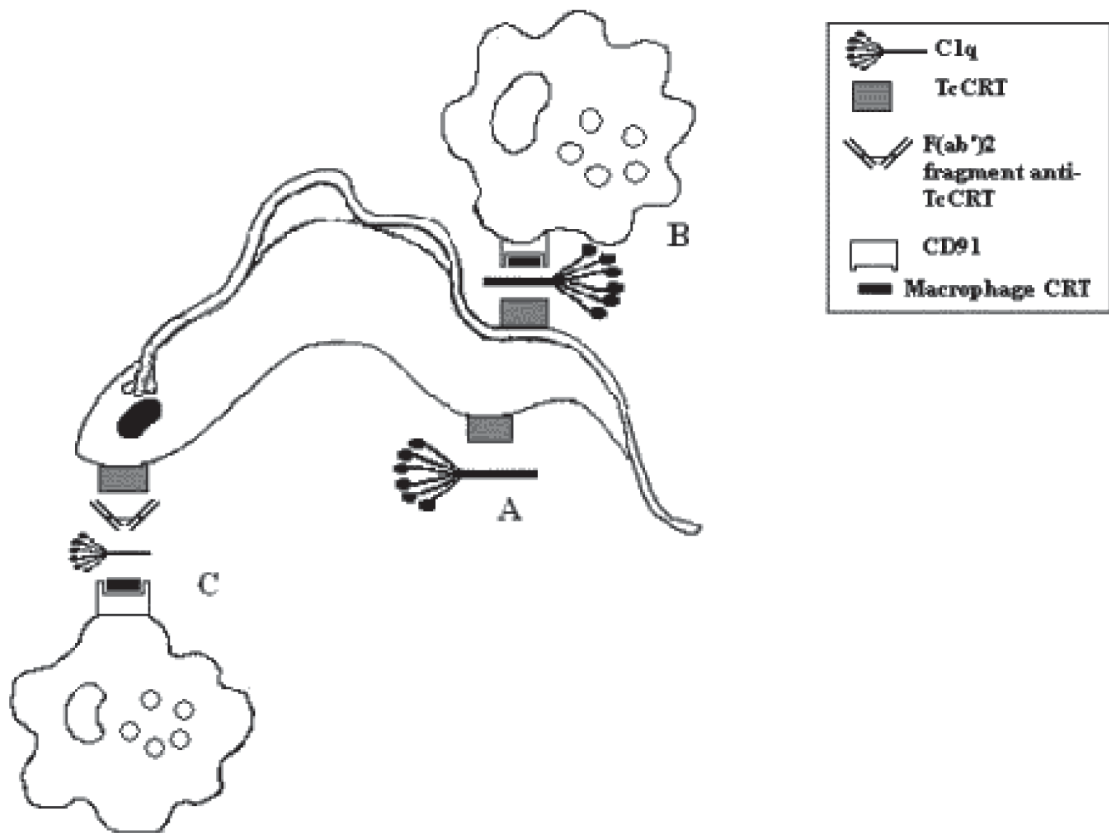
Fig. 4. Possible roles of anti-rTcCRT and anti-TcS F(ab')₂ fragments in the inhibition of *T. cruzi* internalization by host cells. a) Parasite membrane-bound TcCRT specifically interacts with C1q (Ferreira et al., 2004a). b) Possibly, C1q on the parasite membrane is recognized by the CD91-bound host cell CRT, thus facilitating parasite internalization. c) By blocking this interaction, anti-**r**TcCRT and anti-TcS F(ab')₂ fragments could interfere with this internalization process.

facilitating parasite internalization. The interaction between TcCRT and the collagenous portion of vertebrate C1q could be inhibited by F(ab')₂ fragments anti-**r**TcCRT or anti-TcS, thus interfering with the internalization process (C). The *in vitro* results presented in Fig. 3a are compatible with these notions.

The experiments presented here generated necessary information for future *in vitro* and *in vivo* assays, aimed at assessing the role of TcCRT in the trypomastigote capacity to infect different cell types. *In vitro*, this capacity is known to be enhanced by C1q (Rimoldi et al, 1989). Therefore, our demonstration that the C1q / TcCRT interaction can be inhibited *in vitro* by specific antibodies is compatible with the possibility that this interaction can also be inhibited *in vivo*.

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