Reactivity of Chagasic Antigen Antibodies With Noninfected Cells Treated With *Trypanosoma cruzi* Secreted/Excreted Antigens

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Here, we show that antigen antibodies from Chagas' disease patients react with noninfected host cells previously treated with antigens secreted by the trypomastigote forms of *Trypanosoma cruzi*. With the exception of human and Old World monkey cells, which are GAL-negative, cells of all mammals express the GAL epitope (Gal α (1-3)Gal β (1-4)GlcNAc-R) on their surface. Thus only the former ones develop antigen antibodies. Antigen antibodies increase during infection with *T. cruzi*, which expresses GAL epitopes on the surface of the infective forms. Here, we show that incubation of noninfected, GAL-negative cells with antigens shed by *T. cruzi* renders these cells reactive to antigen antibodies purified from chagasic sera. Neither

chagasic sera depleted of antigen antibodies nor antigen antibodies purified from normal sera display reactivity with treated cells. Cell reactivity of chagasic antigen was abolished in the presence of melibiose (Gal α (1-6)Glc) or gal-gal (methyl 3-O- α -D-galactopyranosyl α -D-galactopyranoside). Since shedding of *T. cruzi* antigens can occur in vivo, these antigens may induce reactivity of chagasic antigen with noninfected human cells. The reactivity of noninfected, GAL-negative cells observed only with chagasic antigen antibodies can amplify the range of reactivity of these antibodies and consequently adds to their importance in the pathogenesis of human Chagas' disease. J. Clin. Lab. Anal. 12:108–114, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

GAL epitope, Gal α (1-3)Gal β (1-4)GlcNAc-R, is found in cells from all mammals, with the exception of humans and Old World monkeys (1). Thus only the latter cells develop antigen antibodies, which comprise ~1% of the IgG from normal human sera (1–3).

GAL epitopes are found in internal antigens of epimastigote forms, as well as on surface antigens of trypomastigote and amastigote forms of *Trypanosoma cruzi* (4). These epitopes contribute to the increase of antigen observed in sera from patients with Chagas' disease (2, 3). Antigen antibodies from both chagasic and normal sera bind to Gal α (1-3)Gal β (1-4)GlcNAc – Synsorb or melibiose (Gal α (1-6)Glc) – agarose and react with Gal α (1-3)Gal epitopes of mouse laminin (LAM) (5–10). However, only antigen from patients with acute or chronic Chagas' disease lyse trypomastigotes through different pathways (2, 3, 5–9, 11). Besides contributing to the reduction of parasitemia, chagasic antigen may play a role in

the immunopathology of human Chagas' disease (1, 9, 11, 12). In fact, in in vitro studies, chagasic antigen was found to react with cells infected with *T. cruzi* (4).

T. cruzi secretes/excretes many antigens, including molecules involved in host cell binding (13) such as glycosidases (14), glycosyltransferases (15, 16), and proteases (17–19). Shedding of these molecules also occurs during in vivo *T. cruzi* infection (20, 21). Thus these antigens can either attach to or modify the surface of noninfected host cells, rendering them reactive to antibodies from chagasic sera.

Here, we show that noninfected, GAL-negative cells (Rhesus monkey LLC-MK₂) display antigen reactivity when

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previously incubated with *T. cruzi*-secreted antigens. This reactivity occurs with antigal purified from chagasic but not from normal sera. However, chagasic sera depleted of antigal do not react with these cells, although they react with *T. cruzi*-secreted antigens bound to ELISA plates.

The mechanisms by which *T. cruzi*-secreted antigens induce chagasic anti-gal reactivity with noninfected cells remain to be established. Nevertheless, our data suggest a role for these antibodies in the pathogenesis of human Chagas' disease.

MATERIAL AND METHODS

Antigal and Serum Fractions

Antigal antibodies from normal (N) and chagasic (Ch) pooled sera were purified by affinity chromatography as described (7), with some modifications. Briefly, normal or chagasic (chronic phase) pooled sera were applied to 1 ml columns of packed melibiose-agarose (Sigma, St. Louis, MO), and the eluted sera (void volume) were analyzed or further purified as described below. The columns were then washed with PBS (25 mM Na₂HPO₄, 25 mM KH₂PO₄, 150 mM NaCl, pH 7.2) and the antigal antibodies eluted with 0.1 M glycine buffer, pH 3.0, 150 mM NaCl. These antigal fractions were immediately neutralized in 1 M Tris-HCl, pH 8.0, and their protein content was measured by the method of Bradford (22) and confirmed by the method of Peterson (23). Complete antigal depletion from sera was obtained by incubating eluted sera with rabbit red blood cells (RBC), as described (3). These steps of serum manipulation were carried out in order to employ the antigal depleted sera and the intact sera at the same final dilution.

Epimastigote Alkaline Extract (EAE) and *T. cruzi* Secreted Antigens (Sec Ag)

EAE (polyantigenic alkali extract) was prepared by solubilization of *T. cruzi* epimastigote forms (Y strain) in 0.3 M NaOH, as described by Lissaldo et al. (24). *T. cruzi*-secreted antigens (Sec Ag) were obtained from supernatants of *T. cruzi*-infected host cells, as described (20, 25, 26), with some modifications (6, 27). Four days after infection of 55 cm² monolayer of LLC-MK₂ cells with 5×10⁶ forms of *T. cruzi* trypomastigotes (Y strain), the cells were incubated for 16–18 h in 15 ml fresh M199 containing 2% fetal calf serum. The recovered medium, containing 10 to 20×10⁶ trypomastigotes/ml, was centrifuged at 2,800 ×g for 10 min at 4°C, re-centrifuged at 11,200 ×g, and then filtered through a Millipore membrane (0.22 µm). *T. cruzi* Sec Ag were also prepared in the absence of fetal calf serum in order to determine their protein content (22), which ranged from 30–40 µg/ml. Different batches of Sec Ag were immediately used or stocked in liquid nitrogen.

ELISA

Intact and antigal-depleted sera, as well as purified antigal antibodies, were assayed by ELISA using 2 µg/ml of LAM

(mouse laminin from Engelbreth-Holm-Swarm mouse sarcoma, Sigma), 6 µg/ml of EAE and 3–4 µg/ml of *T. cruzi* Sec Ag as antigens, as described (6, 27). The blocking solution contained 1% ovalbumin (Sigma) and 0.05% Tween 20 in PBS (6, 27). Unless otherwise specified, antigal antibodies from normal and chagasic sera were employed at 50 µg/ml, whereas intact or antigal-depleted sera were diluted 1:200. Peroxidase-labeled antihuman IgG (heavy chain specific, Sigma) was employed at 1:6,000. Inhibition of antigal binding to LAM was assessed by adding 0.2–200 mM gal-gal (methyl 3-O-α-D-galactopyranosyl α-D-galactopyranoside) or melibiose (Galα(1-6)Glc) (Sigma) to sera or purified antibodies.

IFI assays With Host Cells

GAL-negative (Rhesus monkey LLC-MK₂) or GAL-positive (mouse fibroblast L929) cells were seeded at a cell density of 4×10⁴ on chamber slides (NUNC) (28), cultured in M199 containing 5% calf serum, and assayed 24 h thereafter. GAL-negative cells were incubated with *T. cruzi* Sec Ag (30–40 µg/ml) for 24 h at 37°C. After three washes in M199, antigal antibodies or antigal-depleted sera were added and the cells incubated for 30 min at 37°C. Unless otherwise specified, purified antigal antibodies were employed at 50 µg/ml, whereas the final dilution of antigal-depleted sera was 1:200. The reaction was developed using FITC-labeled antihuman IgG (heavy-chain specific, Boehringer, Indianapolis, IN). GAL-positive cells were directly incubated with antigal antibodies and then processed for IF assay as described above. Cell reactivities were scored using a Zeiss photomicroscope equipped with an HBO 100 W/2 lamp. The specificity of the cell antigal binding assay was assessed by adding 0.2 to 200 mM of gal-gal (methyl 3-O-α-D-galactopyranosyl α-D-galactopyranoside) or melibiose (Galα(1-6)Glc) (Sigma) to sera or purified antibodies.

RESULTS

Determination of Reactivity of Intact Sera, Antigal-Depleted Sera and Purified Antigal Antibodies by ELISA

The reactivity of sera and antibodies to *T. cruzi* antigens (Sec Ag and EAE) and mouse laminin (LAM) was analyzed by ELISA. LAM was employed as a source of Galα(1-3)Gal epitope. For comparative purposes, the ELISA data obtained with normal and chagasic intact sera, antigal-depleted sera, and purified antigal antibodies are shown for each of the antigens employed (Fig. 1a, b, c).

Figure 1a shows no differences in the reactivity pattern of 20 µg/ml of either normal or chagasic antigal antibodies with LAM. This reactivity was similar to that obtained with 1:200 diluted intact chagasic sera. Much lower reactivity was observed with intact normal sera. Accordingly, depletion of

Fig. 1. IgG reactivity (ELISA) of intact, antigel-depleted and antigel antibodies from chagasic (Ch) and normal (N) sera with **a** = LAM, **b** = EAE and **c** = Sec Ag (see Materials and Methods). Serum dilution = 1:200, antigel 50 μ g/ml; peroxidase-labeled, antihuman IgG conjugate = 1:6,000. * Antigel diluted at 20 μ g/ml.

antigel antibodies, as judged by the loss of anti-LAM reactivity, was obtained after melibiose chromatography when using normal sera, whereas further absorption with RBC was required when using chagasic sera (data not shown). LAM reactivity with 50 μ g/ml antigel from normal or chagasic sera led to similar OD values higher than 2.0. The reactivity of sera or antigel antibodies to LAM was completely abolished by the addition of competitive sugars (see Material and Methods; data not shown).

Intact and antigel-depleted chagasic sera probed with *T. cruzi* Sec Ag led to high reactivity, which was even higher with EAE bound to ELISA plates (Fig. 1c, b). The reactivity of 1:200 diluted intact and antigel-depleted chagasic sera was about three times higher than with 50 μ g/ml of purified chagasic antigel, when Sec Ag was employed as antigen. The same concentration of antigel purified from normal sera was not reactive with this antigen (Fig. 1c). Antigel antibodies purified from either chagasic or normal sera did not react with EAE (Fig. 1b). Normal sera, depleted or not of antigel antibodies, did not react with either EAE or Sec Ag *T. cruzi* antigens (Fig. 1b, c).

Reactivity of Fractionated Sera and Purified Antibodies With Noninfected Host Cells

Antigel antibodies (50 μ g/ml) purified from chagasic or normal sera reacted with 100% of GAL positive cells, but displayed different IF patterns. Whereas normal antigel showed a discontinuous fluorescent pattern around the entire cell surface, chagasic antigel presented a continuous shell-like layer (Fig. 2a, b). No reactivity was observed when chagasic or normal sera were depleted of antigel antibodies (Fig. 2c and data not shown). Cells incubated only the FITC-labeled antihuman IgG were negative (data not shown).

GAL-negative cells did not react with normal or chagasic sera, or with normal or chagasic antigel antibodies. However, when these cells were previously incubated with *T. cruzi* Sec Ag, antigel purified from chagasic sera reacted with ~30% of these cells. The pattern depicted was a characteristic dotted fluorescence on the cell surface (Fig. 3a). This reactivity was not observed with chagasic sera depleted of antigel antibodies (Fig. 3b). Nevertheless, chagasic sera depleted of antigel reacted with Sec Ag adsorbed to ELISA plates, as described above (Fig. 1c). Antigel purified from normal sera did not react with cells treated with Sec Ag (data not shown). These IF patterns were consistently observed in five separate experiments. The IF reactivity on the cell surface obtained with chagasic antigel was completely eliminated when the antibodies were pre-incubated with either melibiose (Gal α (1-6)Glc) (50 mM) or gal-gal (methyl 3-O- α -D-galactopyranosyl α -D-galactopyranoside) (0.2 mM). Cells incubated with supernatants of noninfected cells or only with FITC-labeled antihuman IgG were also negative (data not shown).

Fig. 2. IFI reactivity patterns of GAL-positive cells (L929) with antigen from normal sera (**a**, 50 $\mu\text{g/ml}$), chagasic sera (**b**, 50 $\mu\text{g/ml}$), and chagasic sera depleted of antigen (**c**, 1:200 dilution). Fluorescein-

labeled, antihuman IgG conjugate = 1:100. **a'**, **b'** and **c'** = phase contrast of **a**, **b** and **c**, respectively.

DISCUSSION

This work shows that *T. cruzi* Sec Ag can induce reactivity of antial antibodies with noninfected host cells. Antibodies with this specificity increase in the acute and chronic phases of human Chagas' disease (3, 5–7). These two chagasic antigen populations are able to lyse trypomastigote forms, although through different mechanisms (3, 5–7; see Table 1). The increase of antigen antibodies in human Chagas' disease probably results from the polyclonal activation of pre-existing GAL-reactive B lymphocytes, as well as a response to *T. cruzi* antigens (9–11). In fact, it is known that only antigen from chronic phase sera, but not from normal sera, induces efficient complement mediated lysis and reacts with GAL-containing F2/F3 trypomastigote purified antigens (7, 8; see Table 1). This indicates that, besides the antigen usually found in normal sera, chagasic sera contain antigen sub-populations of

other fine specificities or affinities, all of them able to bind to melibiose-agarose and mouse laminin, among others (1–10). These and other characteristics of antigen from normal and chagasic sera are summarized in Table 1.

Analysis of ELISA Data for Sera and Antigen Antibodies

In the present study, antigen antibodies are considered to be the population of human antibodies that interacts with the carbohydrate structure $\text{Gal}\alpha(1-3)\text{Gal}\beta(1-4)\text{GlcNAc-R}$, as defined by others (1, 9, 11). Antigen binds to the $\text{Gal}\alpha(1-3)\text{Gal}$ residue of LAM, but can also bind to the $\text{Gal}\alpha(1-6)$ residues of melibiose and interact with $\text{Gal}\alpha(1-2)$ and $\text{Gal}\alpha(1-4)$ residues (1, 9, 11). The population of antigen from normal sera is less heterogeneous than those from patients with several infectious diseases, which in-

Fig. 3. IFI reactivity patterns of GAL-negative cells (LLC-MK₂) pretreated with *T. cruzi* secreted antigens (see Materials and Methods) and incubated with antigel from chagasic sera (**a**, 50 µg/ml), and with chagasic sera depleted of antigel (**b**, 1:200 dilution)*. Fluorescein-la-

beled, antihuman IgG conjugate = 1:100. a' and b' = phase contrast of a and b, respectively. (*) A similar pattern was observed with antigel from normal sera (50 or 75 µg/ml).

TABLE 1. Characteristics of Antigel^a From Normal and Chagasic^b Sera^c

	Normal	Chagasic
1. Reactivity with:		
a. Galα(1-3) Galβ(1-4) GlcNAc-Synsorb	high	high
b. Melibiose (Galα(1-6) Glc) - Agarose	high	high
c. LAM (mainly Galα(1-3) Gal epitope)	high	high
d. α-D-Galp (1-3) Gal	high	high
e. α-D-Galp (1-2) Gal	low	high
f. α-D-Galp (1-6) Gal	low	high
g. α-D-Galp (1-4) Gal	very low	high
h. α-Gal epitope in metacyclic forms	low	high
i. F2/F3 trypomastigote antigens	low	high
2. Ig Isotypes reactive with LAM:		
a. IgM (high in ACS) ^d	low	low
b. IgG (very high in ACS) ^d	high	very low
c. IgA (high in ACS) ^d	absent	absent
3. Affinity with LAM (IgG)	high	high
4. Clonal diversity (specificities)	high	very high
5. Lytic power with trypomastigote forms: ^c		
a. Metacyclic, complement dependent	low	very high
b. Cell derived, complement independent	low	very high
c. Bloodstream, complement independent	low	high (ACS) ^d
6. Amastigote immunohistochemical labeling	absent	present

^aAntibodies reactive with Galα(1-3) Galβ(1-4) GlcNAc-R.

^bFrom the chronic phase, unless specified as ACS^d.

^cData obtained from the references cited.

^dAntigel from acute phase sera.

^eIncreased lytic power with concentrated natural antigel.

clude antibodies induced in response to saccharides expressed by the infective agents (1, 9, 11).

The differences in heterogeneity between chagasic and normal antigel are not reflected on LAM reactivity by ELISA, as shown in Figure 1a. Besides this similar reactivity (1–9, 11), Unterkircher (10) showed that the two antigel populations present the same affinity for LAM.

Two other observations of our ELISA data are also worth mentioning regarding the objectives of the present study. The first is that antigel antibodies from chagasic sera, despite displaying high reactivity with LAM, react poorly with *T. cruzi* Sec Ag and do not react with EAE. This demonstrates that the reactivity of chagasic antigel is restricted to *T. cruzi* molecules poorly represented in Sec Ag and absent in the EAE (Fig. 1). The second observation is that depletion of antigel antibodies from chagasic sera does not substantially modify their reactivity with the *T. cruzi* antigens employed here. In fact, both intact and antigel-depleted chagasic sera react intensively with *T. cruzi* Sec Ag and EAE. These data show that chagasic sera contain anti-*T. cruzi* antibodies reacting with highly immunogenic epitopes other than the antigel reactive ones present in the antigens analyzed here. Thus the reactivity of chagasic sera depleted of antigel with Sec Ag is mainly attributed to *T. cruzi* epitopes other than those reacting with purified antigel.

Only Antigen From Chagasic Sera, But Not From Normal Sera, Reacts With Noninfected Cells Treated With *T. cruzi* Sec Ag

We first verified that the same concentration of antigen purified from chagasic or normal sera displayed different immunofluorescence reactivity patterns with GAL positive cells, as shown in Figure 2a, b. This confirms that antigen antibodies from chagasic sera include antibodies with different characteristics from those present in normal sera, as noted by others (7–10). More importantly, we found that antigen from chagasic but not from normal sera reacts with noninfected, GAL-negative cells pre-incubated with *T. cruzi* Sec Ag (Fig. 3a). Since shedding of trypomastigotes occurs not only in vitro (13) but also in vivo (20, 21), a similar generation of the reactive epitopes could occur in human Chagas' disease.

The different cell IF reactivity patterns observed here with normal and chagasic antigen cannot be attributed to different reactivity to the Gal α (1-3)Gal epitope expressed in LAM, since the same concentrations of antigen from either normal or chagasic sera show similar reactivity with this antigen (Fig. 1a). Besides, whereas even 20 μ g/ml chagasic antigen react with noninfected treated cells, antigen from normal sera shows no reaction even when 75 μ g/ml are employed (data not shown).

Human and Old World monkey cells do not express the GAL epitope Gal α (1-3)Gal β (1-4)GlcNAc-R since the corresponding α -galactosyltransferase gene of these mammals is suppressed (29). However, Souto-Padrón (4), by in situ immunohistochemical studies, demonstrated that chagasic antigen reacted with the surface of Vero cells infected with *T. cruzi*, as well as with antigens apparently released from the parasites. Our results amplify the range of reactivity of these antibodies, since we demonstrate reactivity of chagasic antigen with noninfected cells.

Hypothesis About Origin of Antigen Reactive Epitopes

The simplest interpretation of these data would be that *T. cruzi* Sec Ag are adsorbed on the surface of noninfected cells, leading to chagasic antigen binding. However, no reactivity of antigen-treated cells was observed with chagasic sera depleted of antigen (Fig. 3b). It should be remembered that these *T. cruzi* antigens contain epitopes other than GAL, which are highly immunogenic, since chagasic sera depleted of antigen react strongly with the same antigens bound to ELISA plates (Fig. 1c). Thus if reactivity of chagasic antibodies with noninfected cells results from antigen-binding, this binding should occur mainly with antigens containing GAL or GAL-like epitopes.

Another hypothesis that could explain these data is that antigen reactive epitopes result from some activity other than binding induced by *T. cruzi* Sec Ag on surface molecules expressed by some of the host cells. Galili (31) reported that treatment of human erythrocytes with pronase induced their

reactivity with antigen antibodies. These authors suggested that antigen antibodies may be implicated in the elimination of "altered" human red blood cells. Etienne-Decerf (30) showed that 30% of human thyroid cells, obtained from thyroid primary cultures and treated in vitro with exogenous trypsin, reacted with antigen antibodies. These authors suggested that previous thyroid lesions could expose normally hidden antigenic determinants that would in turn enhance the antigen response, as described to occur after some renal injuries (32). Interestingly, the reactivity pattern of the antigen reactive human thyroid cells obtained by Etienne-Decerf (30) was dotted fluorescence on the cell surface similar to that observed by us in cells reactive with chagasic antigen (Fig. 3a). It is thus conceivable that a similar mechanism could occur with cells treated with parasite proteases containing *T. cruzi* Sec Ag (17–19). Glycosidases and glycosyltransferases, that are also shed by trypomastigotes (15, 33, 34), have been reported to facilitate the action of proteases (35). One may thus speculate that more than one enzymatic activity could be responsible for the GAL or GAL-like epitope generation. In fact, the antigen reactive epitope may not be a true GAL residue, but rather a peptide epitope that may mimic GAL or GAL-like structural conformation (36).

We tried to determine if exogenous proteases (trypsin, pronase, and papain) are able to induce antigen reactivity with noninfected cells. However, all treatments resulted in morphological alterations of the cells. Addition of protease inhibitors of low toxicity (E-64, aprotinin or leupeptin) to *T. cruzi* Sec Ag equally impaired IF analysis of cell reactivity (data not shown).

The mechanism(s) by which *T. cruzi* Sec Ag induce cell reactivity with chagasic antigen remains to be clarified. Nevertheless, our results add new information about a potential role of these antibodies in pathogenesis of human Chagas' disease (4, 9, 11). Whatever its origin, this epitope would be slowly but continuously generated in a micro-environment surrounding externalized trypomastigotes during in vivo infection. This may lead to reactivity of a subpopulation of chagasic antigen with some noninfected host cells, thus contributing to the immunopathology of human Chagas' disease (1, 4). Immunohistochemical studies employing biopsies of human myocardium are planned as a continuation of the present study.

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