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A striking common *O*-linked *N*-acetylglucosaminyl moiety between cruzipain and myosin

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SUMMARY

Single units of O-*linked* N-*acetylglucosamine (GlcNAc), usually components of nuclear and cytoplasmatic proteins, are present at the C-terminal domain of cruzipain (Cz), a lysosomal major antigen from Trypanosoma cruzi. On the other hand, antibodies directed against some self-antigens like myosin are associated with Chagas heart disease. The participation of* O-*GlcNAc moieties in the molecular antigenicity of Cz was determined using GlcNAc linked to aprotinin by ELISA. The immune cross-reactivity between Cz and myosin is mainly focused in the C-T domain. ELISA inhibition assays using rabbit sera specific for Cz and C-T in conjunction with immune-gold electron microscopy analysis of heart tissues from mice immunized with C-T confronted with polyclonal rabbit sera specific for Cz and C-T prior and after myosin adsorption provided evidence which indicates that* O-*GlcNAc moieties constitute a common epitope between Cz and either myosin or other cardiac O-GlcNAc-containing proteins, showing a new insight into the molecular immune pathogenesis of Chagas heart disease.*

Keywords *Chagas disease, cruzipain, myosin,* O-N-*acetyl-*D-*glucosamine,* Trypanosoma cruzi

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INTRODUCTION

Chagas disease, caused by the intracellular parasitic protozoan *Trypanosoma cruzi,* represents an important health problem in Latin America. The Pan American Health Organization (PAHO) estimates that 7·7 million people currently have *T. cruzi* infection in the 21 endemic countries, which have a total population of 532 million people. The PAHO also estimates that approximately 41 200 new vector borne cases of *T. cruzi* infection arise per year and that 14 400 infants are born with congenital Chagas disease annually (1). The disease has also emerged as a public health problem, because of infected travellers carrying the parasites to other regions, in the United States of America, Europe, Australia and Japan (2,3). Chagas heart disease develops in 10–30% of infected individuals and is a common cause of fatal dilated cardiomyopathy (4). There are controversial data on Chagas disease pathogenesis. Independently of the eventual contribution of an autoimmune component (5), pathology has been related to the persistence of *T. cruzi* parasites at the affected organs where they produce a chronic inflammation process (6).

Cruzipain (Cz), a major *T. cruzi* antigen, has been extensively studied as cysteine proteinase and glycoprotein (7) and has been related as candidate for drug target and vaccine development (8). Myosin heavy chains are the most abundant heart proteins, constituting 50% of total protein by weight and recognized in many situations of heart-specific autoimmunity, such as rheumatic heart disease and murine post-Coxsackie B3 autoimmune cardiomyopathy (9). *Trypanosoma cruzi* infection may induce the production of antibodies specific for various cardiac proteins, including myosin (10) as well as cellular immune responses to myosin in humans and mice (11,12). Humoural and cellular cardiac autoimmunity may develop during acute *T. cruzi* infection in the genetically susceptible host (13). There are reports supporting the fact that a molecular mimicry mechanism is responsible for the

development of autoimmunity. Immunization with *T. cruzi* proteins, in absence of live parasites, was shown to induce autoimmunity to self-antigens (14). Immunization of mice with Cz induces expansion of T cells and B cells specific to skeletal myosin and 'autoantibodies' to cardiac myosin (15). On the other hand, immunization with cardiac myosin or *T. cruzi* antigens can induce specific, bidirectional cross-reactive immune responses in the absence of detectable cardiac damage (16). Interestingly, mice immunization with C-T domain has shown that sulfate moieties of Cz molecule are able to elicit specific humoral and cellular immune responses and are involved in the generation of heart tissue damage (17).

O-linked *N*-acetylglucosaminyl (*O*-GlcNAc) moieties constitute an abundant, dynamic reversible and regulatory post-translational modification of serine/threonine residues for numerous cytoplasmic and nuclear proteins (18,19). Recently, it has been suggested that this post-translational modification might be involved in protein–protein interactions but could also modulate the contractile properties of skeletal muscle (20). Many *O*-GlcNAc proteins have been identified to date, and they belong to various classes of proteins including cytoskeletal components, hormone receptors, transcriptional factors, kinases, signalling molecules, nuclear pore proteins and viral proteins, suggesting that *O*-GlcNAc may be implicated in several key cellular systems including transcription, nuclear transport and cytoskeleton structure. Numerous evidences suggest the importance of *O*-GlcNAc in many pathologies including diabetes, cancer, autoimmune and neurodegenerative diseases and also in adaptation processes, underlying its crucial role in cell life (21). Regarding the mentioned pathologies, it was reported that many proteins that are *O*-GlcNAc modified are associated with Alzheimer disease, including tau (22), and clathrin assembly proteins (23). *O*-GlcNAc also plays a role in cancer. In this sense, primary breast carcinomas have decreased levels of *O*-GlcNAc-modified proteins and increased *O*-GlcNAcase activity (24), and the major *O*-GlcNAc attachment site on the proto-oncogene c-Myc is a mutational hot spot for Burkitt's lymphoma (25). In addition, *O*-GlcNAc modification of Elf-1 transcription factor, which results in decreased T-cell receptor zeta-chain gene transcription was found in most patients with systemic lupus erythematosus (26). Moreover, elevation of *O*-GlcNAc levels in 3T3-L1 adipocytes including the *O*-GlcNAc modification of several key proteins in the insulin-signalling pathway directly causes insulin resistance, the hallmark of type II diabetes (18). Furthermore, under the knowledge that abnormal glucose metabolism may contribute directly to depressed cardiac function, specific sites of *O*-GlcNAc modification of myofilament proteins have been identified and provide evidence that this post-translational modification can regulate myofilament function. Recently, site-specific GlcNAcylation of human erythrocyte proteins, differentially regulated in response to glycemic status was suggested to be useful as a diagnostic tool for the early detection of diabetes (27). Considering the adaptation processes, the muscular contraction phenomenon is both dependent on glucose metabolism and highly regulated by phosphorylation/dephosphorylation processes. Moreover, mammalian skeletal muscle fibres display a great potential of adaptation that results from the ability of muscle fibres to adjust their molecular, functional and metabolic properties in response to altered functional demands, such as changes in neuromuscular activity or mechanical loading (28). Using a proteomic approach, fourteen proteins were identified in skeletal muscle as being *O*-GlcNAc modified and further classified in three main classes. One of the contractile proteins includes myosin heavy chain (29).

We have previously demonstrated the presence of *O*-GlcNAc moiety in the C-terminal domain of Cz, a lysosomal proteinase (30). Herein, to evaluate the molecular involvement of *O*-linked GlcNAc moiety present in the major cysteine protease of *T. cruzi*, both in Cz recognition and in the cross-reactivity between Cz and myosin molecules, rabbit cardiac myosin and sera from mice immunized with the complete molecule and with its C-terminal domain were used as tools. In addition, rabbit sera specific to Cz and C-T were also used to test heterologous cross-reactivity on heart tissue of C-T-immunized mice by immunogold electron microscopy.

MATERIAL AND METHODS

Immunization of animals

Balb/c mice immunization protocol was performed as previously described (17). Rabbits (New Zealand white lineage) were immunized with purified Cz extracted from polyacrylamide gels or from C-T domain purified according to Barboza *et al.* (30) (three doses of 50 μg protein intradermal via, in each case). All experimental procedures were conducted in the INP, 'Dr Mario Fatala Chaben', Ministerio de Salud, Argentina, in accordance with the ethical legislations and regulatory entities, established in Argentina and International Guides for care and use of laboratory animals. Sera from rabbit immunized with Cz and C-T were obtained as described (17).

Coupling of sugar GlcNAc to aprotinin

Aprotinin (30·7 nmol), glucosamine (1·54 μmol) and glutaraldehyde (1·54 μmol) were mixed during 18 h at

room temperature with magnetic stirring. The resultant solution was further dialysed with distilled water, concentrated and analyzed by ultraviolet laser matrix-assisted desorption/ionization time-of-flight mass spectrometry (UV-MALDI-TOF-MS).

Dot blot assays

Rabbit muscle myosin (3 μg) was confronted with sera from mice immunized with Cz and C-T followed by anti-mouse total IgG (D) and anti-mouse IgG1, 2a, 2b and 3 (E) coupled to alkaline phosphatase and developed with Enhanced Chemiluminescence (ECL) reagent (Amersham Biosciences, Cambridge, UK) by quantitative dot blot assays. The quantization was performed with a FujiLAS1000 densitometer equipped with IMAGE GAUGE 3.122 software (Fuji Film, Tokyo, Japan). The total recognition of myosin by mice sera specific for Cz was considered 100%.

ELISA and ELISA inhibition assays

ELISA assays were performed using 1 μg of Cz or rabbit muscle myosin as antigen. Rabbit polyclonal sera specific for *T. cruzi* microsomal fraction for purified Cz and for *Tc*13 were tested in comparable titres (1/1000) in comparison with preimmune sera. In ELISA inhibition assays, Cz and myosin (1 μg each) were confronted separately with constant amounts of polyclonal rabbit sera specific for Cz and C-T post-adsorption with growing amounts of *O*-GlcNAc as inhibitor (sera tested in the same title 1/6400) for 1·5 h at 37°C in the presence of 0·1 M beta-mercaptoethanol were incubated with equal volumes of increasing amounts of soluble GlcNAc (0–75 μM, 2 h at 37°C). The percentage of inhibition was determined by comparing serum reactivity in the presence and absence of the inhibitor. Preimmune sera values were discounted. The results represent at least three experiments with data performed as triplicates.

Immunegold electron microscopy analysis

Cardiac tissue from mice immunized with C-T was fixed and processed for immune-gold electron microscopy analysis and confronted with sera from rabbit immunized with either Cz or C-T prior and after adsorption with myosin and BSA, respectively (30 μL of rabbit anti-Cz and anti-C-T sera were separately adsorbed twice each with 10 μg of myosin from rabbit muscle and incubated 2 h at 37°C. Once adsorbed, the sample sera was diluted 1/5, centrifuged, and supernatant was used). Controls were performed by similar adsorption protocol with BSA. Spot counting was performed using 80 fields ×10 000 magnification using ImageJ software (available from http://rsb.info.nih.gov/ij/), and results were expressed as spot number/field.

Statistical analysis

Values are given as means ± SD of triplicate determinations from a minimum of three separate experiments. Pair comparison between groups was evaluated with one-way analysis of variance, followed by Tukey test in GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). A value of $P < 0{\cdot}05$ was considered significant.

RESULTS AND DISCUSSION

Evidence of immunological cross-reactivity between cruzipain and myosin

Evidence of cross-reactivity between both molecules was obtained using sera specific for Cz and other Cz containing parasite fractions or nonrelated parasite antigens. With the aim of getting deeper into the immune cross-reactivity between myosin and Cz, polyclonal rabbit sera specific for Cz, for antimicrosomal fraction and for *Tc*13, were confronted with Cz and myosin as antigens. Cz was recognized by its cognate sera and by sera specific for antimicrosomal fraction but in a significant lower level, no recognition was observed with either sera specific for *Tc*13, a nonrelated parasite antigen or preimmune sera (Figure 1a). Although sera specific for Cz recognized myosin, the recognition was significantly lower with myosin compared with Cz, and no recognition was observed neither with control sera nor with sera specific for the nonrelated parasite antigen *Tc*13, confirming cross-reactivity between both molecules (Figure 1b). Our findings are in line with a previous report showing that antibodies specific for Cz were capable to bind to a 223-kDa antigen from a mouse heart extract, identified by sequencing analysis as the mouse cardiac myosin heavy chain and once purified by immunoadsorption, recognized to this molecule, suggesting cross-reactive epitopes between Cz and myosin (15).

Cross-reactivity between cruzipain and myosin is mainly focused in the C-terminal domain of cruzipain

The alignment of protein sequences between Cz and mouse myosin heavy chain does not display a significant homology between both molecules. However, it has been described by protein sequencing analysis that they share three short common linear peptide sequences and there is some cross-reactivity between them (15). The Cz linear

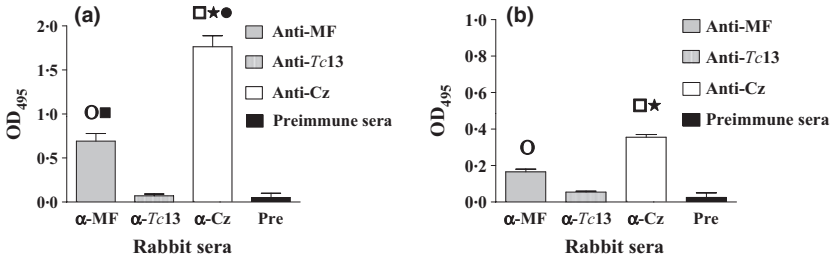

Figure 1 Evidence of cross-reactivity using sera specific for cruzipain (Cz) and other Cz containing fractions or nonrelated parasite antigen. ELISA plates were coated with either 1 μg of Cz (a) or myosin (b) and confronted with rabbit sera specific for an antimicrosomal fraction from *Trypanosoma cruzi* (anti-MF), anti-*Tc*13 antigen or sera specific for Cz, using dilutions according to comparable titres. The bars represent the mean ± SD from triplicate determinations. The results presented are representative of three independent experiments. Analysis by Tukey test revealed in a and b: $^{\circ}P < 0{\cdot}05$ vs. anti-*Tc*13 and preimmune (Pre) control sera; $^{\square}P < 0{\cdot}01$ vs. anti-*Tc*13 and Pre; $^{\star}P < 0{\cdot}05$ vs. anti-MF. In (a), $^{\blacksquare}P < 0{\cdot}01$ vs. anti-MF (b); $^{\bullet}P < 0{\cdot}01$ vs. anti-Cz (b).

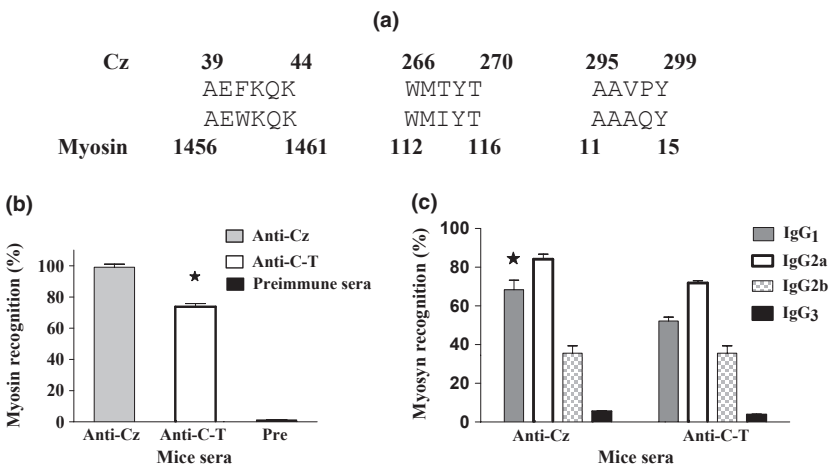

Figure 2 Cross-reactivity between cruzipain (Cz) and myosin is mainly focused in the C-terminal domain of Cz. The Cz linear peptidic sequences corresponding to the residues 39–44, 266–270 and 295–299 have high homology with the myosin sequences 1456–1461, 112–116 and 11–15, respectively, according to described by Giordanengo *et al.* (15). Sequences containing residues 266–270 and 295–299 are located in the C-T domain. (a) Percentage of rabbit muscle myosin recognition by sera from BALB/c mice immunized with Cz ($n = 5$) or C-T ($n = 5$) compared with preimmune sera. Sera total IgG levels (b) or IgG1, 2a, 2b and 3 isotype (c) levels specific for Cz were determined by ELISA. Myosin recognition percentage was calculated. Values are presented as the mean of myosin recognition (%) ±SD. The OD mean value for myosin recognition with sera specific for Cz was considered 100%. In (b), $^{\star}P < 0{\cdot}05$ vs. anti-Cz; in (c), $^{\star}P < 0{\cdot}05$ vs. IgG1 (anti-C-T). The results presented are representative of three independent experiments.

sequences corresponding to the residues 39–44, 266–270 and 295–299 have high homology with the myosin sequences 1456–1461, 112–116 and 11–15, respectively, as can be observed in Figure 2(a). Two of them, those containing residues 266–270 and 295–299 are located in the C-T domain. When multiple sequence alignment of myosin protein sequences is performed, it can be shown that the linear homologue peptidic epitopes containing threonine in position 114, 116 or 117 is shared between myosin's from different origin and Cz.

In addition, total IgG levels cross-reactive with rabbit myosin muscle were measured in sera from mice immunized with Cz and C-T. The percentage of myosin recognition obtained in sera of immunized mice with Cz and C-T demonstrates that the cross-reactive epitopes between Cz and myosin are mainly located in the C-terminal domain of the molecule (Figure 2b). To determine the isotypes involved in the immune cross-reactivity between both proteins, the isotype profile was evaluated. The analysis of the isotype profile of both mice sera specific for Cz and C-T revealed that the IgG response to myosin was mainly IgG2a followed by IgG1 and IgG2b, while IgG3 was almost undetectable (Figure 2c). Accordingly, analysis of sera from mice immunized with Cz revealed the highest

reactivity against myosin with the IgG2a isotype, which could lead to complement fixation, inflammation and subsequent pathology in the heart tissue (15). In this sense, IgG2a is reported as the main isotype involved in the production of autoantibodies in the chronic stage of *T. cruzi* experimental infection (31) and in the development of anti-self B-cell responses in autoimmune prone mice lacking functional Fas or FasL (32).

Whereas IgG1 levels showed a significant difference when measured in sera from mice immunized with Cz and C-T, IgG2a and 2b isotype levels showed similar values in both immunized groups indicating that IgG2b and 2a isotypes, involved in the cross-reactivity, are directed to epitopes from the C-T domain. The use of mice sera immunized with purified Cz or its native C-T domain showed that the immune cross-reactivity between Cz and myosin is mainly focused in the C-T domain. Strikingly, the presence of this common epitope between myosin and Cz is described in little domains of the same molecular mass (25 kDa) in both cases, located in the heavy chains motor domains of myosin and in the C-T domain from Cz.

Heterologous cross-reactivity between cruzipain and myosin and other cardiac proteins in fibre and mitochondria from heart tissue of C-T-immunized mice

Taking into account that C-T might contain cross-reactive epitopes with myosin and other cardiac *O*-linked-GlcNAc-containing proteins, the cross-reactivity between these molecules was also assessed by immunogold analysis. To achieve this goal, sera from rabbit immunized with either Cz or C-T prior and after myosin adsorption were confronted with heart tissue from mice immunized with C-T in comparison with nonimmunized control mice. As expected, heart control mice fibres showed cross-reactivity by immune-gold with both rabbit polyclonal-specific sera. After discounting spots corresponding to nonimmunized control mice, half of the reactivity observed in fibres and mitochondria with sera immunized with Cz disappeared when confronting with myosin-adsorbed sera, indicating that the adsorption with myosin has blocked mainly the cross-reactive epitopes between Cz and myosin, as well as the presence of proteins containing other different cross-reactive groups in cardiac tissue (29) (Figure 3a), confirming the immune cross-reactivity between Cz and myosin and/or other *O*-GlcNAc-containing proteins. In addition, the similar value of spots obtained with sera specific for Cz or C-T reinforce the relevance of the C-T in the immune cross-reactivity of Cz with myosin and other *O*-GlcNAc-containing proteins. It is worth noting that heterologous immune-gold reactivity spots could be also observed in cardiac mitochondrial proteins from

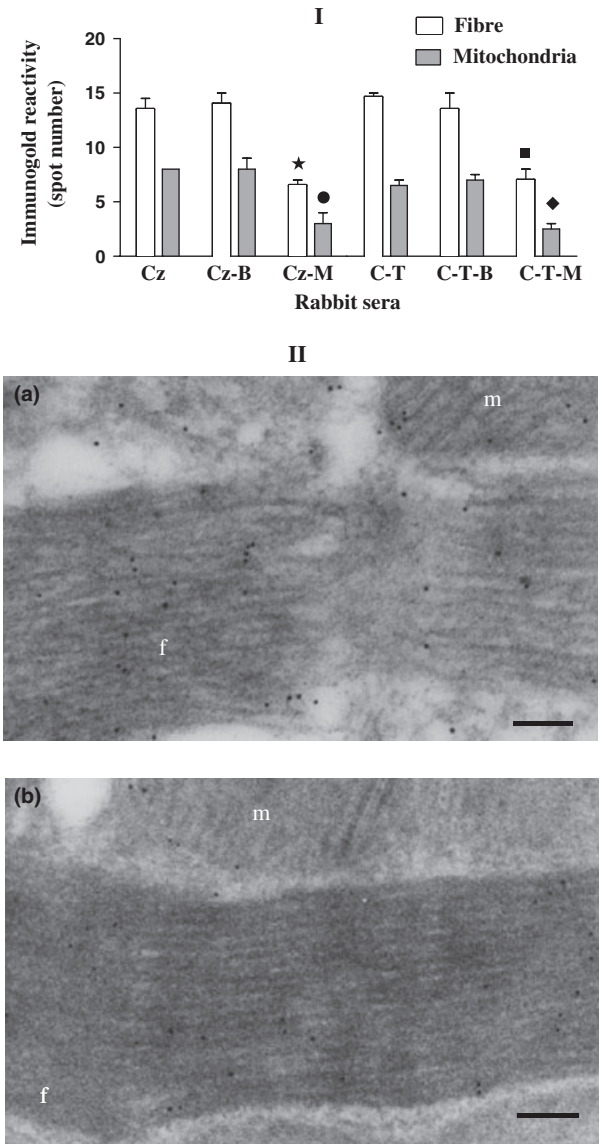

Figure 3 Heterologous cross-reactivity between cruzipain (Cz) and myosin and other cardiac proteins in fibre and mitochondria from heart tissue of C-T-immunized mice. (I) Analysis was performed by counting spots of immunegold reactivity between serum from rabbit immunized with either Cz or C-T prior and after adsorption with myosin and heart tissue from mice immunized with C-T. Adsorption controls using BSA were performed. It is worth mentioning that nonimmunized control mouse displaying no specific labelling was discounted. ★, ■$P < 0{\cdot}05$ vs. rabbit sera specific for Cz and C-T without (Cz, C-T) and with (Cz-B, C-T-B) adsorption with BSA (in fibre), respectively; •, ▲$P < 0{\cdot}05$ vs. rabbit sera specific for Cz and C-T without (Cz, C-T) and with adsorption with BSA (Cz-B; C-TB), (in mitochondria), respectively. (II) Immune histochemistry of cardiac tissue sections from C-T-immunized mice. Heart sections from immunized mouse confronted with mice sera specific for Cz, prior (a) and after (b) myosin adsorption. Bars: (a and b) represent 200 nm. f, fibre; m, mitochondria.

C-T-immunized mice when tested with rabbit sera specific for Cz and C-T. Recently, Hu *et al.* (33) have determined that specific mitochondrial proteins are *O*-GlcNAcylated in rat cardiomyocytes, and an increase in this modification contributes to impaired mitochondrial function.

Cardiac ultrastructure abnormalities were found in C-T-immunized mice as previously described (17). Figure 3(b) shows the microphotographs from immunegold electron microscopy to contrast findings reported in the manuscript along with spot counting presented in Figure 3(a). Interestingly, immune-reactive gold particles are observed in either fibre or mitochondria of heart from C-T-immunized mice. In addition, it can be observed that the immunegold reactivity decreased significantly when heart tissue was confronted with myosin-adsorbed sera specific for Cz as compared to the obtained with sera specific for Cz prior to the adsorption.

Involvement of *O*-GlcNAc moieties in both the immune recognition of cruzipain and the cross-reactivity between Cz and myosin

To evaluate the involvement of *O*-GlcNAc moiety in the antigenicity of Cz molecule, GlcNAc was coupled to aprotinin. A dose-dependent recognition was observed both with polyclonal rabbit sera specific for Cz and C-T, indicating that these moieties are antigenic (Figure 4a). The direct recognition of the GlcNAc linked to aprotinin on the plates performed with the specific polyclonal antibody directed to the C-T domain was slightly higher than that obtained with antibodies directed to the whole molecule, demonstrating that these epitopes are antigenic and located in the C-T domain. In addition, the inhibition of Cz recognition was also tested by adsorption of sera with growing amounts of GlcNAc. Sera specific for C-T showed the highest inhibition percentage (30%) with 10 μM of inhibitor. However, only about half value of inhibition percentage (15%) was obtained using the same concentration of inhibitor to block polyclonal serum specific for Cz as compared with the inhibition obtained with the sera specific for C-T (Figure 4b). The inhibition of the specific recognition of these groups by anti-C-T was higher than that obtained by the serum specific for Cz, probably due to the main presentation of these epitopes in the C-T domain and confirming the antigenicity of *O*-GlcNAc.

To further evaluate the involvement of these moieties in the cross-reactivity between Cz and myosin, inhibition of myosin recognition was obtained confronting this molecule with polyclonal rabbit sera specific for Cz and C-T, previously adsorbed with GlcNAc (Figure 4c). Inhibition ELISA assays performed with myosin as antigen and GlcNAc as inhibitor showed that growing concentrations of

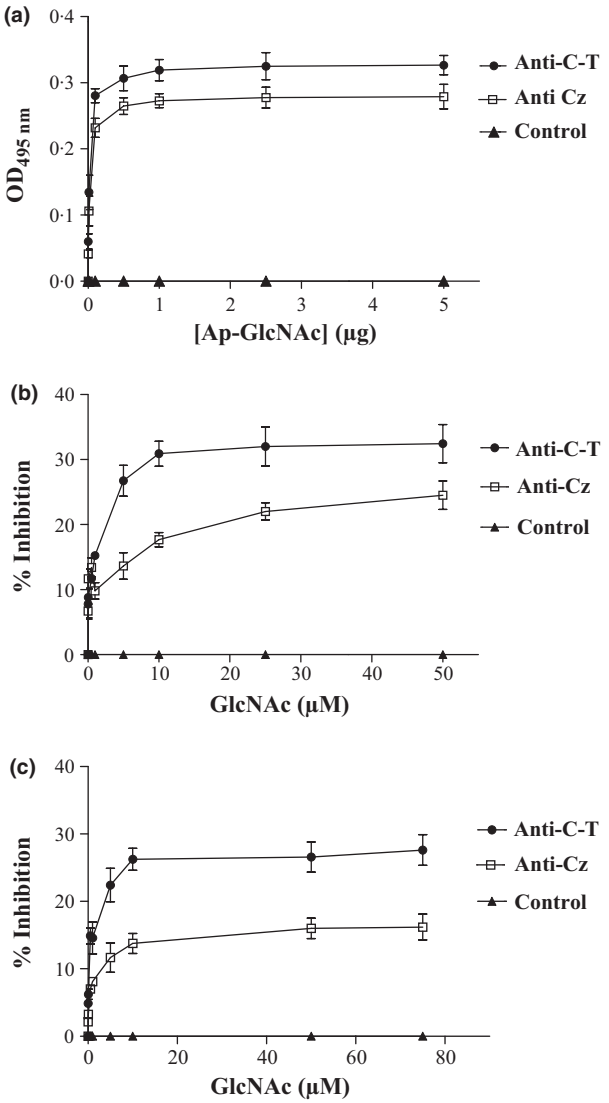

Figure 4 Involvement of *O*-GlcNAc moieties in both the immune recognition of cruzipain (Cz) and the cross-reactivity between Cz and myosin. To evaluate the involvement of *O*-GlcNAc moieties in the immune recognition of C-T domain by its cognate antisera, *O*-GlcNAc coupled to aprotinin was obtained in a specific single step reaction. The results demonstrate the participation of this epitope in the immune recognition of Cz (a). *O*-GlcNAc inhibition of Cz and myosin immune recognition by polyclonal rabbit sera specific for Cz and C-T. ELISA assays were performed using Cz (b) or rabbit muscle myosin (c) as antigen. Each antigen was confronted with sera from rabbit immunized with Cz and C-T. Constant amounts of each serum (1/6400) were incubated in the presence of 0·1 M beta-mercaptoethanol with equal volumes of increasing amounts of soluble GlcNAc. The percentage of inhibition was determined by comparing serum reactivity in the presence and absence of the inhibitor. The results represent means ± SD of duplicate determinations from at least three independent experiments.

this carbohydrate epitope were able to block partially the cross-reactivity between this contractile protein and sera specific for Cz and C-T. A 25% of inhibition raised with anti-C-T confirmed the participation of *O*-GlcNAc epitope in the cross-reactivity between myosin and the C-terminal domain. Using a serum specific for Cz, previously adsorbed with growing amounts of *O*-GlcNAc, the inhibition of myosin recognition (10%) resulted lower than the obtained with anti-C-T reinforcing that the cross-reactivity because of *O*-GlcNAc epitopes between these molecules is mainly focused in the C-T domain. The modification of Ser and Thr residues of proteins with an *O*-*N*-acetylglucosamine is an essential and dynamic post-translational modification of metazoans. Deletion of the *O*-GlcNAc transferase, the enzyme that adds *O*-GlcNAc, is lethal in mammalian cells highlighting the importance of this key post-translational modification in regulating cellular function. In addition, molecular mimicry between some pathogens and host has been proposed as a mechanism for the development of autoimmune-like diseases (34). Similarly, environmental and genetic factors are involved in the development of rheumatic carditis and inflammatory heart disease, as a result of mimicry between the group A from streptococcus and heart (35). Noticeable, *O*-GlcNAc is the immunodominant epitope of the group A streptococcal carbohydrate. T-cell-dependent antibody response to the dominant epitope of streptococcal polysaccharide, *N*-acetylglucosamine, is cross-reactive with cardiac myosin. In the case of the streptococcal group A carbohydrate epitope, *N*-acetyl glucosamine structurally mimics cardiac myosin in the human disease, rheumatic carditis, and in animal models immunized with streptococcal M protein and cardiac myosin (36).

It is well known that antibodies directed against some self-antigens like myosin are associated with inflammatory heart diseases such as rheumatic carditis, myocarditis and Chagas disease dilated cardiomyopathy (37). Owing to the fact that molecular mimicry is a hallmark of the pathogenesis of the mentioned heart diseases and knowing that single units of this *O*-glycosidic modification, although they are usually components of nuclear and cytoplasmatic proteins, are atypically present in Cz as well as in cardiac proteins, the antigenicity of *O*-GlcNAc group in the C-T domain from a lysosomal parasite protease, Cz, and its antigenic cross-reactivity with myosin were examined, demonstrating that this moiety constitutes a common epitope between Cz and myosin and other cardiac GlcNAc-containing proteins. In summary, our findings clearly provide evidence that indicates the participation of *O*-linked GlcNAc moieties present in the C-T domain of Cz both in the molecular antigenicity of Cz and in the cross-reactivity between GlcNAc-containing molecules from host heart tissue and parasite providing a new insight to better understand the molecular pathogenesis of Chagas heart disease.

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