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Sulfates are main targets of immune responses to cruzipain and are involved in heart damage in BALB/c immunized mice

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Abstract

***Trypanosoma cruzi*, the agent of Chagas disease contains a major cysteine proteinase, cruzipain (Cz), with an unusual carboxyl-terminal extension (C-T). We have previously reported the presence of sulfate groups in the N-linked oligosaccharide chains of this domain. In order to evaluate the immune responses to sulfated moieties on Cz, BALB/c mice were immunized with purified Cz and C-T prior and after desulfation treatment. The humoral immune response to sulfates on Cz or C-T was mainly IgG2b. Interestingly, the abolishment of IgG2b reactivity when desulfated antigens were used as immunogens demonstrates that esterified sulfate groups are absolutely required for eliciting IgG2b response to Cz. Sera from chronically *T. cruzi*-infected subjects with mild disease displayed higher levels of total IgG and IgG2 antibodies specific for sulfated epitopes compared with those in more severe forms of the disease. A significant reduction of C-T-specific delayed-type hypersensitivity reaction in C-T-immunized mice was observed when desulfated C-T was challenged, suggesting the involvement of sulfate groups in the generation of memory T-cell responses. Moreover, immunization with C-T in the absence of infection elicited ultrastructural abnormalities in heart tissue. Surprisingly, hearts from sulfate-depleted C-T-immunized mice did not present pathological alterations. This is the first report showing that sulfate-bearing glycoproteins from trypanosomatids are able to elicit specific humoral and cellular immune responses and appeared to be involved in the generation of heart tissue damage. These results represent a further step in the understanding of the role of Cz in the course of *T. cruzi* infection.**

Introduction

The American trypanosomiasis, Chagas disease, constitutes a major health problem in Central and South America, affecting 16–20 million people and with more than 100 million exposed to the risk of infection (1). This disease occurs in three forms: acute, indeterminate and chronic phases. In contrast to the acute phase in which parasitemia is high, in the chronic phase parasites are extremely low in blood. Around 30% of infected individuals become chronic and develop progressive heart or digestive disease long term after the initial infection (2).

Trypanosoma cruzi, the causative agent of Chagas disease, is an intracellular parasitic protozoan that contains a major cysteine proteinase (CP), cruzipain (Cz) (3). This lysosomal enzyme, as all type I CPs from trypanosomatids studied so far (4), bears in addition to a catalytic moiety with high homology to other enzymes belonging to the papain family a carboxyl-terminal extension (C-T) which at variance with other enzymes of this type is retained in the mature protein. This unusual domain contains a number of

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post-translational modifications and is responsible for the immunodominant antigenic character of Cz in natural and experimental infections (5–7). Although the bulk of the enzyme is lysosomal, some plasma membrane-bound isoforms of CPs immunologically cross-reactive with Cz were evidenced in the different developmental stages of *T. cruzi* (8, 9). In addition to these differences in localization, atypical isoforms non-adsorbed to ConA-Sepharose with different carbohydrate content were also described (10). Metacyclic trypomastigotes have been reported to be able to release CPs, involving most probably Cz among them into the medium (11, 12).

Previous studies have shown that Cz may induce autoimmune responses in experimental models, protective immunity against experimental *T. cruzi* infection as well as cellular immune responses mediated by IFN-gamma-producing cells (13–15). Human and murine infections with *T. cruzi* elicit a strong humoral and cellular immune response to Cz (6, 13, 14, 16–18). Moreover, we showed that the severity of the disease in chronic chagasic patients was associated with high levels of Cz-specific antibodies (19).

Glycoconjugates such as glycolipids and glycoproteins have been widely described in *T. cruzi*. Sulfated lipids are present on the surface of *T. cruzi* as part of glycolipidic structures (20, 21). We have recently reported the presence of sulfated N-linked oligosaccharides as components of Cz, the major lysosomal glycoprotein of *T. cruzi* (22). The aim of the present study is to determine whether these sulfated structures on Cz are immunogenic. To achieve this goal, we compared the humoral immune responses elicited by immunization of BALB/c mice with desulfated cruzipain (*d*Cz) or desulfated carboxyl-terminal extension (*d*C-T) with those elicited by unaltered Cz and C-T, respectively. We also assessed the humoral immune responses to sulfate groups on Cz in the sera of patients with chronic *T. cruzi* infection.

Herein, we demonstrate that esterified sulfate groups are absolutely required for eliciting IgG2b response to Cz in BALB/c mice. These structures are involved in the human humoral immune response to Cz, particularly in the early stages of the natural course of chronic *T. cruzi* infection. Delayed-type hypersensitive (DTH) assays suggest the involvement of sulfate groups in the generation of memory T-cell responses. Moreover, we show, for the first time, that immunization with C-T is capable to generate ultrastructural pathological effects in the heart in the absence of infection. The abrogation of these abnormalities when sulfate groups are depleted from C-T supports the participation of sulfates in the induction of tissue damage.

Methods

Parasites and culture

Epimastigotes of *T. cruzi*, Tulahuen strain, Tul 2 stock, were grown in axenic medium, harvested and washed with 0.25 M sucrose and 5 mM KCl as previously described (23).

Purification of antigens

Cz was purified from epimastigotes of *T. cruzi*. Briefly, epimastigotes were disrupted by three cycles of freezing at −20°C and thawing. The freeze-thawed parasites were extracted and centrifuged as previously described (24). The cell-free extract was precipitated (50%) with saturated ammonium sulfate solution and the pellet was dissolved in 10 ml of 50 mM Tris–HCl pH 7.6 containing 150 mM NaCl followed by dialysis against the same buffer. The first step of the purification was affinity chromatography on ConA–Sepharose (24). The active elute was dialyzed against 50 mM Tris–HCl, 1 mM EDTA pH 7.6 and loaded on a Mono Q HR^{TM} 10/10 anion exchange column equilibrated with the same buffer in an fast performance liquid chromatography system as described by Duschak *et al.* (19) with some modifications to separate Cz isoforms from contaminant proteins. The elution was performed using a non-lineal NaCl gradient with an isocratic step at 180 mM NaCl monitored by absorbance at 280 nm. Fractions were collected at a rate of 1 ml min^{-1} and the active fractions on contaminant serinecarboxipeptidase (SCP) were assessed at 25°C by monitoring the hydrolysis of furyl acryloyl-Phe-Phe-OH (FA-Phe-Phe-OH) spectrophotometrically at 330 nm (25). Active fractions of Cz on benzoyl-Pro-Phe-Arg-para-nitroanilide (Bz-PFA-*p*NA) were pooled, dialyzed against Tris–HCl 10 mM pH 7.6 (26) and concentrated by SAVANT. Inactivation of Cz was performed with 20 μM *trans*-epoxy-succinyl-L leucyl-amido-4-guanidine butane (E-64) for 1 h on ice.

The C-T domain was obtained by self-proteolysis of highly purified Cz active preparations in sodium acetate buffer pH 6.0 at 38°C for 48 h and purified by gel filtration in a Biogel P-30 column (1.5 × 100 cm) eluted with Tris–HCl buffer pH 7.6 containing 50 mM NaCl. Elution was monitored by measuring absorbance at 280/230 nm. A sample of each fraction was analyzed by SDS–PAGE followed by silver staining or electroblotting, using an anti-Cz polyclonal antibody for developing (26).

Desulfation treatments

Enzymatic desulfation was performed by digestion with sulfatase from *Abalone entrails* (Type VII; Sigma–Aldrich Co., St Louis, MO, USA) (25 mU) in 50 mM sodium acetate pH 5.0 for 18 h at 37°C. For chemical desulfation, samples were passed through 0.5 ml of AG50W-X8 resin (H^+) and the column washed with water (2 ml). After the addition of pyridine (0.015 ml), the sample was lyophilized, dissolved in dimethylsulfoxide:methanol (9:1 v/v, 0.2 ml), adjusted to pH 4 with dilute HCl, heated at 100°C for 2 h and freeze dried (27).

Immunization of BALB/c mice

Female BALB/c mice (6–8 weeks old; five animals per group) were injected by subcutaneous via with E-64-inactivated purified Cz and inactive purified C-T, both chemically [*d*Cz(s), *d*C-T(s)] and enzymatically [*d*Cz(e), *d*C-T(e)] desulfated, and emulsified with IFA. The immunization protocol consisted in a five weekly doses of 10 μg of each immunogens per mouse per dose. Control groups were injected with PBS containing reactive for solvolysis or sulfatase treatment, respectively, and were processed in the same condition than the sulfated or desulfated immunogens.

All experimental procedures were conducted in accordance with guidelines for care and use of laboratory animals of the Instituto Nacional de Parasitología, 'Dr Mario Fatala Chaben', Ministerio de Salud, Argentina.

Study population

The diagnosis of Chagas disease is currently assessed by indirect hemagglutination, indirect immunofluorescence and enzyme immunoassay using whole homogenates of the epimastigote form of *T. cruzi* as antigen (28). An individual is considered infected if at least two out of the three tests were positive. Sera from chronic chagasic patients with different degrees of cardiac dysfunction as determined by Kuschnir grading system (29) were provided by the Laboratory of Parasitology from Hospital Eva Peron, Buenos Aires, Argentina. Group 0 (G0, n = 11) comprised seropositive individuals showing a normal chest X-ray and a normal electrocardiography (ECG); group 1 (G1, n = 5) seropositive patients had a normal chest X-ray but abnormalities in the ECG and group 3 (G3, n = 13) seropositive patients had ECG abnormalities, heart enlargement and clinical or radiologic signs of heart failure. The uninfected control group consisted of age-matched individuals who were found to be negative for *T. cruzi* by serologic testing. Chagas disease patients and uninfected individuals with hypertension, vascular, ischemic or congenital heart disease, cancer, syphilis, HIV, diabetes, arthritis or serious allergies were excluded from the present study.

Patient's sera

Blood to be used for serum component analysis was obtained from patients and control subjects by venipuncture, allowed to coagulate at 4°C and centrifuged at 1000 × g for 15 min. Non-hemolyzed serum was separated, and aliquots were stored at −70°C until use.

Determination of total IgG and IgG subclasses in mice and human sera

Antibodies specific to purified Cz or C-T were determined by an indirect ELISA procedure prior and after desulfation treatment of antigens as described previously (19). In brief, plates were coated with 5 μg ml^{-1} of control (untreated) or purified *d*Cz or *d*C-T, blocked and incubated with a 1:100 or 1:50 dilution of mice and human sera, respectively. Specific total IgG was detected by incubation with biotinylated horse anti-mouse IgG (H+L) with a 1:2000 dilution (Vector Laboratories, Inc., Burlingame, CA, USA). Specific IgG isotypes were detected by incubation with a 1:100 dilution of goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (PharMingen, BD Biosciences) and biotinylated mouse anti-human IgG2 and IgG4 mAbs subclasses (PharMingen, BD Biosciences) at a final concentration of 3.5 μg ml^{-1} followed by incubation with HRP–streptavidin (Vector Laboratories, Inc.). The reaction was developed with *o*-phenylenediamine dihydrochloride and stopped with sulfuric acid. Samples were assessed in duplicate and plates were read at 490 nm with an ELISA reader (MR 700, Dynatech Laboratories, Alexandria, VA, USA). The percentage of the remnant reactivity of human sera to Cz was calculated as = [$OD_{490\ nm}$ to *d*Cz/$OD_{490\ nm}$ to untreated Cz] × 100.

Dot blot assays were performed using the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories) following the manufacturers instructions (Bio-Rad Bulletin, 1141, 1984). Briefly, 50 μl per spot of 2 μg ml^{-1} untreated and *d*Cz or *d*C-T were seeded in triplicate and sera from mice immunized with Cz or C-T were added at a 1:100 dilution followed by incubation with a biotinylated horse anti-mouse IgG (H+L) diluted 1:2000 or biotinylated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 at a 1:100 dilution, followed by an anti-mouse HRP–streptavidin diluted 1:1000 and visualized using ECL (Amersham) enhanced chemiluminescence's reagent. Spots were visualized using a FujiLAS1000 densitometer equipped with IMAGE GAUGE 3.122, software, Fuji Film, Japan.

SDS–PAGE with or without gelatin

Purification was followed by 10% SDS–PAGE using the discontinuous buffer system described by Laemmli (30) and minigels were stained with silver nitrate (31). Detection of proteinase activity of purified Cz was performed in 10% resolving SDS–acrylamide gels containing 0.15% co-polymerized gelatin as previously described by Duschak *et al.* (32). Protein content was measured by Bradford's method (33).

Western blotting

Samples submitted to SDS–PAGE were electrotransferred to nitrocellulose membranes for 2 h at 200 mA. After blotting, nitrocellulose sheets were cut into strips and post-coated with tris-buffered saline solution containing 3% non-fat power milk (TBS-M). Sera from immunized mice were added at 1/100 dilution followed by incubation with goat biotinylated anti-mouse IgG (H+L) (Vector Laboratories, Inc.) or biotinylated anti-mouse IgG1, IgG2a, IgG2b and IgG3. After washing, membranes were incubated with HRP–streptavidin. Color development was done with a 4-chloro-1-naphthol (Sigma Chemical Co., San Louis, MO, USA) in methanol/PBS solution and stopped with distillate water.

Measurement of DTH

Twenty-five days after the last immunization, purified C-T with or without desulfation treatment, were intra-dermally injected in the footpads of C-T- or *d*C-T-immunized mice. BSA was injected in the opposite pad as control. C-T and *d*C-T DTH was quantitated by a standard pad-swelling assay. Pre-challenge pad thickness was measured with a micrometer (Mitutoyo).

Immunoelectron microscopy analysis

Mice immunized with Cz and C-T prior and after desulfation treatment and control groups immunized with either sulfatase or solvolysis reagents were sacrificed 7 days after the last immunization. Mice were anesthetized, bled by eyeball puncture for serological studies and killed by cervical dislocation. Complete autopsies and histopathological analysis were performed on immunized and control mice. Ultrastructural features were studied in hearts from immunized and control mice. Hearts were fixed by immersion in 3% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, for 24 h at low temperature. After washing by immersion in 0.32 M sucrose containing washing solution for 24 h, fixed tissues were diced into small blocks, followed by 1.5% OsO4 in the same buffer. After dehydration in graded alcohol solutions, the specimens were embedded in epoxi resins embedding medium kit Fluka for 72 h at 60°C. Thin sections were sliced in a Porter-Blum MT2 Sorvall ultramicrotome and the sections

picked up in 300-mesh single hold grids. Staining was done first with uranil acetate and then with Reynolds lead citrate stain. The specimens were examined under a Siemens Zeiss C10 (Siemens, Germany) electron microscope. Photographs were taken with a Kodak electron imaging film (Eastman Kodak, Rochester, NY, USA).

Statistical analysis

Comparisons of the levels of total IgG or IgG isotypes prior and after desulfation treatment in mice or different clinical patient groups were evaluated by Wilcoxon test (Mann–Whitney *U*) and *t*-test for independent samples, respectively. Differences were considered to be statistically significant at $P < 0.05$. Repeated measures analysis of variance was used to compare net pad swellings in DTH reactions, followed by a simple effect test to study the interactions. When the interactions were significant, the Turkey test for multiple comparison procedure with $\alpha = 0.05$ was used.

Results

Purification and characterization of Cz and C-T

Cz eluted from ConA–Sepharose affinity columns might contain high-mannose-rich glycoprotein contaminants of similar apparent molecular mass. To avoid this contamination, we purified Cz including an additional Mono Q purification step (19) with some modifications. The use of a Mono Q column anion exchange chromatography by application of an isocratic step showed two peaks of elution, named P_I and P_{II} (Fig. 1A). Fractions from P_I showed activity on FA-Phe-Phe-OH, confirming that they contain SCP and also a low activity on Bz-PFA-*p*NA due to remnant Cz activity. Fractions from P_{II} (200–500 mM) only showed activity on Bz-PFA-*p*NA indicating that P_{II} contains a highly purified preparation of Cz (Fig. 1A). The analysis by SDS–PAGE and silver staining of fractions belonging to P_I and P_{II} confirmed the purity of Cz in P_{II} fractions (Fig. 1B). The inactivation of purified Cz was performed with E-64, an irreversible inhibitor of CPs, and the inhibition effect was confirmed by SDS–PAGE gelatin-containing gels (Fig. 1C). Purified Cz was also submitted to self-proteolysis and the C-T domain was purified by separation from remnant intact Cz or inactive intermediate fragments using Biogel P30 as previously described (26). The purity of C-T was shown by silver stained SDS–PAGE (Fig. 1D). Purified inactivated Cz and inactive C-T were submitted to chemical and enzymatic desulfation treatment in order to be used as immunogens.

Humoral and cellular immune responses to sulfate moieties on Cz in immunized BALB/c mice

In order to evaluate humoral and cellular immune responses to sulfated structures on Cz, BALB/c mice were immunized with either purified Cz devoid of enzymatic activity or purified C-T, in both cases prior or after chemical or enzymatic desulfation treatment. As previously reported, Cz was able to induce high levels of total IgG antibodies specific to Cz. Antibody levels were as high as those obtained from Tulahuen strain, Tul 2 stock (Tul 2)-infected mice (Fig. 2A). IgG levels specific to Cz measured in mice immunized with

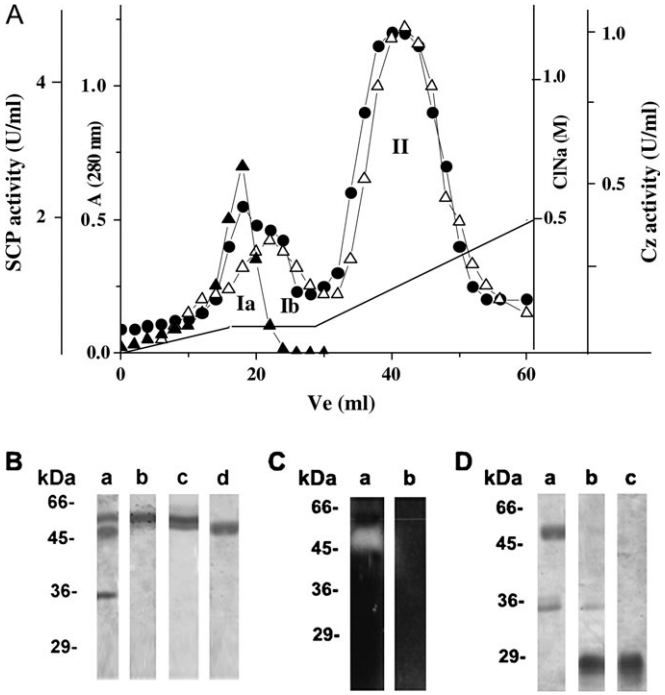

Fig. 1. Purity and inactivity of native Cz isoforms and C-T domain. (A) Native Cz isoforms from *Trypanosoma cruzi* were purified by chromatography on a Mono Q™anion exchange column of the ConA-Sepharose eluate. Cz activity (open triangles) was assessed with Bz-PFA-*p*NA and SCP activity (closed triangles) was measured by monitoring the hydrolysis of FA-Phe-Phe-OH. Protein was determined as A280 nm (closed circles). Peak II fractions were pooled and dialyzed against Tris–HCl 10 mM pH 7.6. (B) Samples obtained from different steps of the purification procedure were analyzed on 10% silver-stained SDS–PAGE: 5 μg ConA-Sepharose eluate (a); 3 μg Mono Q fraction Ia (b); 3 μg Mono Q fraction Ib (c) and 2 μg Mono Q pool II Peak (d). (C) Activity gel of purified Cz without (a) or with E-64 inactivation treatment (b). (D) Self-proteolysis of purified Cz and purification of C-T domain by Biogel P30–SDS–PAGE and silver staining of purified Cz before self-proteolysis (a), intermediate fragment and C-T after self-proteolysis (b) and purified C-T after Biogel P30 (c).

sulfate-depleted Cz did not differ from those found in mice immunized with untreated Cz. Conversely, Cz-specific IgG levels were significantly lower in *d*C-T-immunized mice compared with those recorded in C-T-immunized animals (Fig. 2A). Similar results were obtained when either chemical or enzymatic methods were employed for desulfation. SDS–PAGE and western blot analysis confirmed the immunogenicity of sulfated moieties on C-T (Fig. 2B). It is worth noting that the protein profile of Cz and C-T chemically or enzymatically sulfate-depleted samples did not differ from non-treated samples when compared by SDS–polyacrylamide gels (data not shown). UV MALDI-TOF-MS analysis showed that after desulfation, N-linked oligosaccharides remain unaltered (22).

In order to determine whether immunization with these sulfate-bearing structures could elicit a specific cellular immune response, DTH was measured 24 and 48 h after inoculation with complete or desulfated C-T in the footpad of immunized and non-immunized control mice. By 24 h after inoculation of C-T in mice immunized with C-T, a positive cutaneous reaction was induced (Fig. 2B). Interestingly, although not

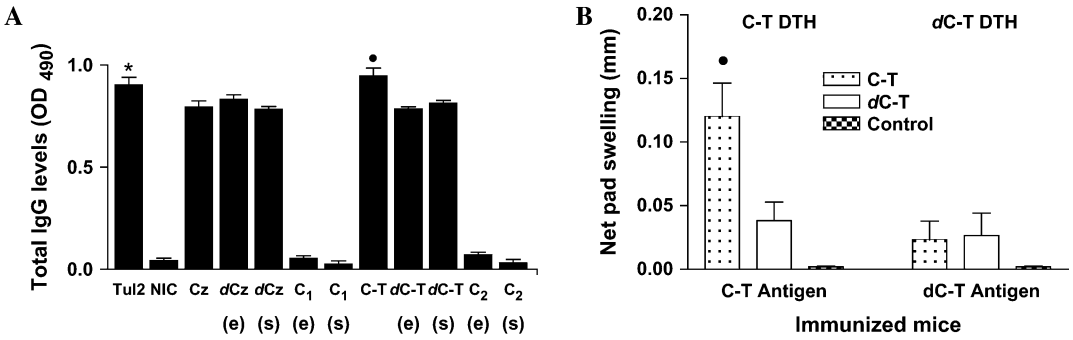

Fig. 2. Humoral and cellular immune responses induced by immunization with Cz and C-T prior and after desulfation treatment. (A) Mice groups (n= 5) were infected with blood trypomastigotes from *Trypanosoma cruzi*, Tulahuen strain, Tul 2 stock (Tul 2) or immunized either with Cz or C-T, prior or after enzymatic [*d*Cz(e), *d*C-T(e)] or chemical [*d*Cz(s), *d*C-T(s)] desulfation treatment. C_1(e) and C_2(s): enzymatic and solvolysis treatment control groups corresponding to *d*Cz and *d*C-T, respectively. NIC: non-infected control. Sera IgG levels specific for Cz were determined by ELISA. *P<0.0001 versus non-infected control; •P <0.05 versus *d*C-T(e) and P<0.01 versus *d*C-T(s). The results presented are representative of three independent experiments. (B) Footpad swelling was measured after local inoculation of purified C-T prior and after desulfation treatment in four mice immunized with complete C-T antigen. C-T- and *d*C-T-specific DTH reactions were measured. C-T-immunized mice and five PBS–CFA-immunized control mice. Values are presented as the mean net pad swelling value ±SD of four mice per group. Data represent mean values from three independent experiments. •P<0.05 by Turkey test when comparing inoculation of untreated antigen with desulfated C-T antigen and with non-immunized control mice 24 h after inoculation.

abrogated, the DTH reaction significantly decreased after inoculation of these mice with *d*C-T. The involvement of sulfated groups in the generation of memory T-cell responses was further confirmed by the low cutaneous reaction observed in mice immunized with *d*C-T. Inoculation of C-T or *d*C-T in control mice did not induce positive responses (Fig. 2B). The footpad indurations decreased to levels comparable to those found in non-immunized controls at 48 h after inoculation (data not shown).

Profile of IgG isotypes in mice immunized with Cz or C-T after desulfation treatment

The analysis of the isotype profile revealed that the IgG response to Cz was mainly IgG1 followed by IgG2b and IgG2a, while IgG3 was undetectable (Fig. 3IA and IB). Likewise, C-T elicited both IgG2b and IgG2a (Fig. 3IIA and IIB). Interestingly, the total IgG responses to sulfate moieties either on Cz or C-T were of the IgG2b isotype as demonstrated by the abolishment of the IgG2b response after desulfation treatment and the lower levels of IgG2b specific to Cz elicited upon immunization with *d*Cz or *d*C-T compared with those induced by the complete cognate antigens (Fig. 3I and II). By contrast, increased IgG1 levels were not associated to sulfated epitopes whereas IgG3 antibodies were not detectable (Fig. 3IA and IB). The IgG2a profile differs from the other isotypes; while desulfation treatment did not affect the recognition to Cz in Cz-immunized mice, a significantly lower level of IgG2a specific to Cz was obtained in response to *d*Cz than in mice immunized with the untreated antigen (Fig. 3IA and IB). These observations suggest a partial participation of sulfate groups in the IgG2a response to Cz. In addition, when *d*Cz was used as immunogen, IgG2a response was significantly higher in comparison to that obtained with the complete molecule, indicating that desulfation treatment might expose new epitopes (Fig. 3IB and IIB). Whereas the presence of IgG1 antibodies specific to Cz and C-T in the sera of immunized mice was confirmed by western blot analysis (data not shown), we were unable to detect IgG2a and 2b isotypes. However, their presence was revealed by dot blot assay, suggesting that denaturizing of samples under SDS–PAGE conditions (such as boiling and reducing) could be responsible for modifications of conformational epitopes. The pattern of recognition for the different isotypes observed by dot blot assays confirmed previous ELISA results, showing the complete disappearance of the C-T recognition on sulfate-depleted samples (Fig. 4). Sulfate moieties are also antigenic in the natural course of *T. cruzi* infection, as a lower reactivity of *d*C-T compared with untreated C-T was found in sera from chronically *T. cruzi*-infected mice.

Ultrastructural pathological changes in BALB/c mice immunized with C-T

Previous reports have involved Cz in the induction of tissue damage (13, 17, 34, 35). In order to determine whether sulfate structures are involved in these pathological changes, tissue specimens from mice immunized with sulfate-depleted C-T were analyzed and compared with those from mice immunized with complete C-T.

Longitudinal sections of heart tissue from control mice showed normal aspect with myocardial fibers characterized by sarcomers with a classic pattern of cross-striation, limited by ordered lines of mitochondria (Fig. 5A). In C-T-immunized mice, tissue abnormalities were induced in heart, showing growth of interfibrillar space, dissociation of myofibers with electrodense material in both myofibers and mitochondria (Fig. 5B). Contrarily, mice immunized with sulfate-depleted antigen displayed the typical organization of heart tissue without any pathological alteration (Fig. 5C).

Humoral response to sulfate structures in chronic chagasic patients

The striking involvement of sulfate moieties in the humoral immune response to Cz in mice prompted us to evaluate

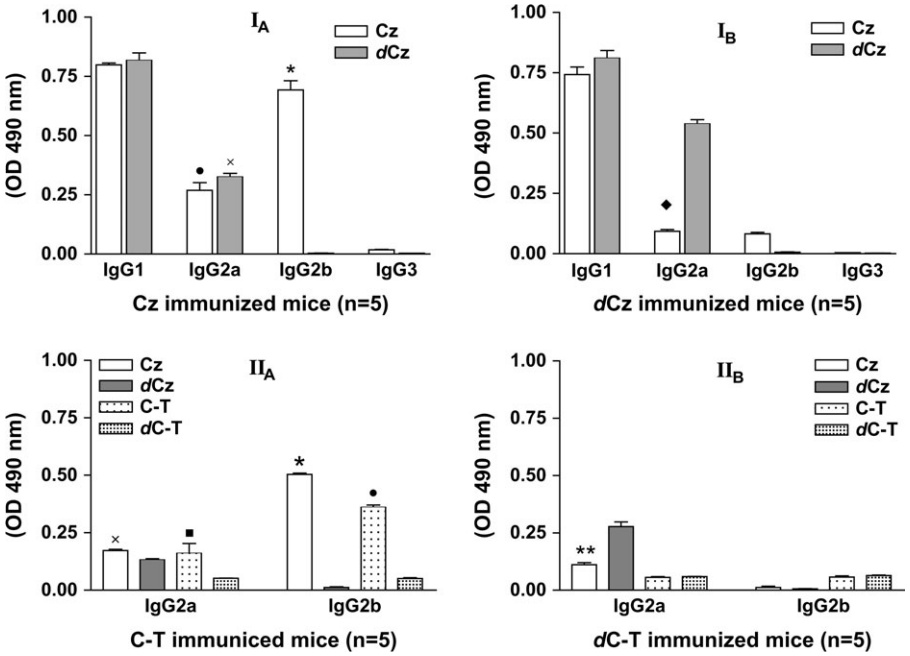

Fig. 3. Profile of IgG isotypes in mice immunized with Cz or C-T after desulfation treatment. (I) Specific IgG1, IgG2a, IgG2b and IgG3 levels were measured by ELISA in sera from mice immunized with Cz (IA) or chemically *d*Cz (IB), using Cz or *d*Cz as antigens. Each bar represents the mean OD ±SD for the different IgG isotypes. **P*= 0.0286 versus IgG2b *d*Cz (A), *P*= 0.0006 versus IgG2b Cz (B); •*P*= 0.0134 versus IgG2a Cz (B); ×*P*<0.0001 versus IgG2a *d*Cz (B); ♦*P*<0.001 versus IgG2a *d*Cz (B). (II) Levels of specific IgG2a and IgG2b in mice immunized with C-T (IIA) or chemically *d*C-T (IIB). C-T-specific IgG isotypes were assessed by ELISA in sera pools from immunized mice as described in Methods. Similar results were obtained in three independent experiments. **P*<0.001 versus IgG2b *d*Cz (A), *P*<0.0001 versus IgG2b Cz (B); •*P*<0.001 versus IgG2b *d*C-T (A), *P*<0.0001 versus IgG2b C-T (B); ×*P*= 0.0009 versus IgG2a *d*Cz (A), *P*= 0.0009 versus IgG2a Cz (B); ▪*P*= 0.0286 versus IgG2a *d*C-T (A); ***P*= 0.001versus IgG2a *d*Cz (B).

whether these sulfated oligosaccharide structures on Cz were targets of the humoral immune response in the natural course of chronic *T. cruzi* infection in humans. Interestingly, our results showed that patients in early stages of chronic *T. cruzi* infection display positive total IgG and IgG2 antibody responses to sulfate groups on Cz, with half of the sera from subjects in the G0 and G1 groups loosing more than 50% of the reactivity to this glycoprotein after desulfation treatment (Fig. 6). Conversely, humoral immune responses to sulfated moieties on Cz in patients with severe cardiac dysfunction are lower than that observed in patients with less severe forms of the disease. IgG1 levels did not vary among the different clinical groups evaluated prior and after treatment (Fig. 6).

Discussion

The antigenicity and immunogenicity of Cz in murine models as well as in human Chagas disease have been extensively described (13, 14, 17, 18, 36, 37). Cz has been also related with parasite metabolism (38) and identified as both an important candidate for trypanocidal drug design (39, 40) and for vaccine development (14). However, the involvement of the substituted glycans of this protein in the molecule antigenicity has been scarcely explored. The determination of its particular immunogenic region would be of relevance for the diagnosis and immunoprophylaxis of *T. cruzi* infection. Herein, we demonstrate that esterified sulfate groups are absolutely required for eliciting IgG2b responses to Cz and they are also capable to induce T-cell responses in BALB/c mice. Moreover, we show that subjects chronically infected with *T. cruzi* mount specific humoral immune responses to this glycoprotein.

In *T. cruzi*, sulfated structures have been described as part of glycolipids, representing common antigens on the surface of the parasite and mammalian cells (20). We recently described the presence of sulfated high-mannose-type oligosaccharides on the unique *N*-glycosylation site of the C-T domain (Asn 255) as a new striking feature in this glycoprotein (22). The presence of sulfate groups in N-linked oligosaccharides has been reported in virus (41) and especially in mammalian cells (42–44) and these groups have been mainly implicated in several specific molecular recognition processes (45, 46). Our results in conjunction with those reported in *Dictyostelium discoideum* (47) are, so far, the only ones describing the antigenic properties of these structures. Moreover, this is the first report describing the antigenic properties of sulfated N-linked oligosaccharides in glycoproteins from trypanosomatids.

In the present study, we have applied a modified purification procedure to obtain highly purified Cz from a parasite lysate, combining a ConA-Sepharose column followed by an additional step of anionic exchange chromatography including an isocratic step to eliminate a co-eluting SCP previously identified by Parussini *et al.* (25). The literature has shown some contrasting data related to the immunopathological effects of Cz depending on the immunogen being native or

recombinant (13, 14, 18). However, our findings using purified Cz as immunogen show that purified native Cz induces pathological effects in the heart of BALB/c immunized mice (data not shown). However, the induction of immunopathological effects due to the active peptidase contaminant, present when Cz preparations obtained by applying a single ConA-Sepharose affinity purification process, cannot be ruled out.

In *T. cruzi*-infected mice as well as in the human infection, IgG1 and IgG2 subclasses have been reported as the prevalent anti-parasitic responses (48–50). The total IgG levels obtained from mice immunized with either Cz or C-T were comparable to those found in Tul 2-infected mice, reinforcing the antigenic relevance of this glycoprotein in the humoral immune response to *T. cruzi*. However, the immunogenicity of sulfate groups was only manifested when *d*C-T was used as antigen, probably due to the high representation of these epitopes in the C-T domain (22). IgG response to Cz resulted mainly IgG1 followed by IgG2b and IgG2a, while IgG3 was undetectable. The high levels of IgG1 obtained even after desulfation indicates that this isotype is not associated to sulfated epitopes (Fig. 3). Contrarily, both the abolishment of the IgG2b response after desulfation treatment in BALB/c mice immunized with untreated antigens and the striking decrease in IgG2b responses to Cz when *d*Cz or *d*C-T were used as immunogens compared with the levels induced by the complete cognate antigens revealed, for the first time, that IgG2b responses specific to Cz are exclusively elicited toward sulfated groups in the C-T. Taking into account that IgG2b is one of the predominant isotype responses to Cz, sulfated moieties might be crucial in eliciting the humoral immune response to this glycoprotein. Likewise, sulfated groups on Cz are partially involved in the IgG2a response to this molecule.

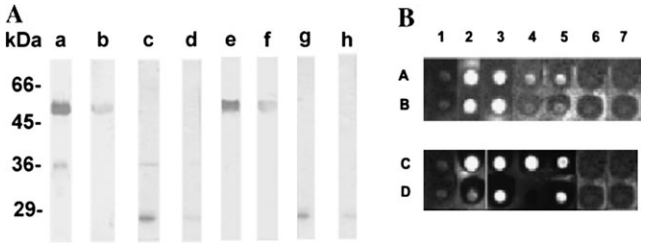

Fig. 4. Immune recognition of sulfated moieties on native Cz or C-T in immunized or chronically *Trypanosoma cruzi*-infected mice. (A) Analysis of the immune recognition to sulfated epitopes by western blotting. Three micrograms of purified Cz and C-T prior (lanes a and c) or after (lanes b and d) chemical desulfation, respectively, were loaded and run in SDS gels, electrotransferred to nitrocellulose strips and probed with pooled sera from mice immunized with Cz (lanes a and b) or with C-T (lanes c and d). Cz (lanes e and f) and C-T (lanes g and h) were confronted with sera from mice immunized with either Cz (lane e) or desulfated Cz (lane f) and with C-T (lane g) or desulfated C-T (lane h), respectively. (B) Two micrograms of purified Cz or C-T prior (A and C) or after (B and D) chemical desulfation treatment, respectively, were loaded (in lane 6, antigens were also submitted to denaturizing with SDS plus heat treatment), probed with pooled sera from mice immunized with Cz (A and B, lanes 3–7) or C-T (C and D, lanes 3–7) and revealed with anti-IgG1 (lane 3), IgG2b (lane 4), IgG2a (lanes 5 and 6) and IgG3 (lane 7). Total IgG responses to sulfated moieties were assessed in sera from uninfected (lane 1) and chronically *T. cruzi*-infected (lane 2) mice.

The intrinsic biochemical characteristics of Cz or alternatively, regulatory factors released as part of the immune response as well as the genetic background of the mice strain may influence the isotypic pattern of Cz-specific antibodies. Since IL-4 and IL-5 production are associated to IgG1 (51), the above described isotypic distribution in favor of IgG1 is in accordance with the notion that Cz may drive the host immune response toward a Th2 profile (14). Taking into account that the susceptibility to *T. cruzi* infection has been related with a Th2 profile (14) and that susceptible murine strains display an enhanced IL-4 production (52), Cz could play a key role for parasite survival in the host during *T. cruzi* infection (53). Furthermore, it had been demonstrated that the transfer of IgG2a and IgG2b antibodies could protect from lethal murine infection with the parasite (48). Our findings showing that chronically *T. cruzi*-infected subjects with less severe clinical forms display higher levels of total IgG and IgG2 antibodies to sulfated groups might indicate their potential role in the control of chronic *T. cruzi* infection. In this regard, a higher frequency of *T. cruzi*-specific memory T-cell responses had been associated with a better

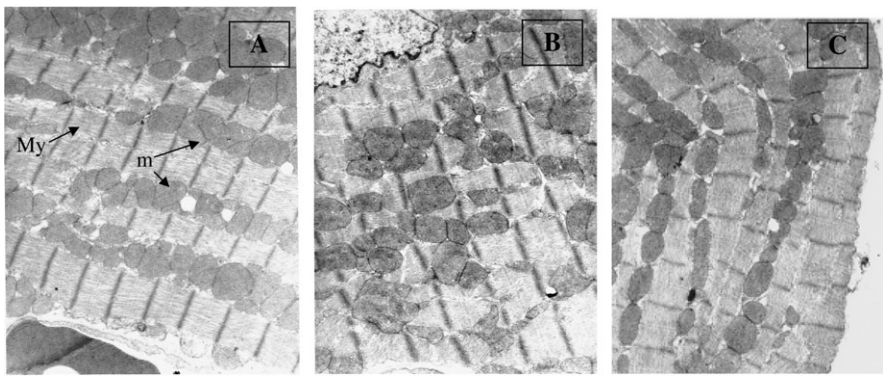

Fig. 5. Cardiac ultrastructural features from mice immunized with C-T and *d*C-T. Morphological analysis was performed by transmission electron microscopy on mice heart tissue. Longitudinal heart sections (×6300) from (A) control mouse showing normal pattern; (B) C-T-immunized mouse showing severe abnormalities and (C) normal pattern similar to controls in mice immunized with *d*C-T. Results are representative of three mice per group, in two independent experiments. My (myofibers) and m (mitochondria) are signed by arrows.

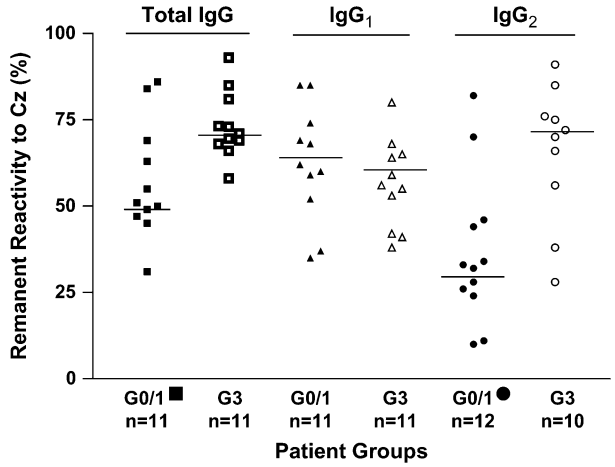

Fig. 6. Humoral immune response to sulfated epitopes specific to Cz in chronic Chagas disease patients. ELISA plates were coated with Cz or *d*Cz and confronted with sera from chronic Chagas disease patients with different degrees of cardiac dysfunction. Each point represents the mean percentage of the remnant reactivity to Cz after desulfation treatment for each patient as described in Methods. Total IgG, IgG1 and IgG2 levels were measured in three independent experiments. Absorbance at 490 nm measured with untreated Cz was considered as 100% of the reactivity to Cz. ▪*P*= 0.0286 versus total IgG G3; ●*P*= 0.0155 versus IgG2 G3.

clinical status of chronic Chagas disease patients (54, 55). That *T cruzi*-specific antibodies may also have beneficial effects against disease progression in the chronic phase cannot be ruled out, as suggested by the protective effect of antibodies during chronic HIV infection (56). In human infection with *Plasmodium falciparum*, high IgG2 levels were also associated to an efficient protective immunity (57). It is worth noting that the humoral immune response to sulfated structures on Cz found in chronically *T. cruzi*-infected mice further supports the immunogenicity of these groups in the natural course of *T. cruzi* infection. On the other hand, the significant reduction of the C-T-specific DTH induced by challenge with *d*C-T in mice immunized with C-T suggests the involvement of sulfate groups in the generation of memory T-cell responses, as DTH reaction is absolutely dependant on the presence of memory T cells, either CD4+ or CD8+ (58–60).

This is the first study, to our knowledge, showing that C-T, the antigenic domain from Cz, used as immunogen in absence of *T. cruzi* infection, is capable to generate ultrastructural abnormalities in the heart. The abrogation of these alterations when sulfate groups were depleted from C-T supports the involvement of sulfated structures in the induction of tissue damage. Taking into account that Cz is a high-mannose-type glycoprotein, high-mannose receptor contains two portions with distinct carbohydrate recognition properties with the ability to bind sulfated and non-sulfated oligosaccharidic chains (61) and mannose receptors are expressed by antigen-presenting cells in non-lymphoid peripheral organs including cardiac muscle in mice (62), we could envision that sulfate groups in the C-T domain might interact with target cells via the mannose receptor eliciting the consequent tissue damage.

In conclusion, this is the first report showing that sulfate-bearing glycoproteins in trypanosomatids are targets of specific humoral and cellular T-cell responses and appeared to be involved in the generation of heart tissue damage. Our findings represent a further step in the understanding of the role of Cz in the course of *T. cruzi* infection.

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Abbreviations

Bz-PFA-pNA	benzoyl-Pro-Phe-Arg-para-nitroanilide
Cz	cruzipain
CP	cysteine proteinase
C-T	carboxyl-terminal extension
*d*C-T	desulfated carboxyl-terminal extension
*d*Cz	desulfated cruzipain
DTH	delayed-type hypersensitive
ECG	electrocardiography
E-64	trans-epoxy-succinyl-L leucyl-amido-4-guanidine butane
FA-Phe-Phe-OH	furyl acryloyl-Phe-Phe-OH
SCP	serinecarboxipeptidase

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