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Original article

DNA immunization with the ribosomal P2β gene of *Trypanosoma cruzi* fails to induce pathogenic antibodies

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Abstract

Patients with chronic Chagas' heart disease (cChHD) develop a strong IgG response against the C-terminal region of the *Trypanosoma cruzi* ribosomal P2β protein (TcP2β). These antibodies have been shown to exert an in vitro chronotropic effect on cardiocytes through stimulation of the β1-adrenergic receptor (β1-AR). Moreover, the presence of antibodies recognizing the TcP2β C-terminus was associated with cardiac alterations in mice immunized with the corresponding recombinant protein. Here, we demonstrate that DNA immunization could be used to modulate the specificity of the anti-TcP2β humoral response in order to avoid the production of pathogenic antibodies. After DNA injection, we detected IgG antibodies that were directed only to internal epitopes of the TcP2β molecule and that did not exert anti-β1-AR functional activity, measured as an increase in intracellular cAMP levels of transfected COS-7 cells. Accordingly, DNA-immunized mice did not present electrocardiographic alterations. These data demonstrate that anti-TcP2β antibodies elicited by DNA immunization are completely different in their specificity and functional activity from those produced during *T. cruzi* infection.

Keywords: *Trypanosoma cruzi*; DNA immunization; Ribosomal P2 protein

1. Introduction

Chronic Chagas' heart disease (cChHD) results from infection with the protozoan *Trypanosoma cruzi* and is one of the most important causes of heart failure in Latin America. Cardiac involvement typically appears decades after initial infection and leads to arrhythmia, cardiac failure, thromboembolic phenomena, and sudden death. Histological examination of the heart reveals inflammatory foci, destruction of cardiac fibers, and marked fibrosis affecting multiple areas of the myocardium [1].

The pathogenesis of cChHD has been discussed extensively over the last decades [2–5]. The failure of conventional histological methods to find parasites in blood and tissues led to the statement that the late clinical manifestations of the disease occur in the absence of the parasite. This has been the main support for the autoimmune hypothesis to explain the origin of cardiac damage in cChHD. Nevertheless, sensitive immunohistochemical techniques and polymerase chain reaction (PCR) amplification enabled the detection of parasitic antigens [6] and DNA [7–9] in hearts of patients with cChHD. Moreover, the combination of data obtained from murine and human infections indicated that the severity of the disease is directly associated with the presence of the parasite in cardiac tissue (rev. in [5]). These findings suggest that heart parasitization is most likely the principal stimulus for the perpetuation of myocardial inflammation and for the generation of a strong anti-parasite response that can lead to autoimmune injury of the heart tissue by cross-reacting T and/or B cells [2]. In particular, there are a growing number of reports suggesting the pathogenic effect of antibodies from patients with cChHD on heart cell functions [10–14].

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These alterations have been shown to be electric and mechanical and seem to result from the binding of IgG antibodies to β-adrenergic and M2-cholinergic receptors on the myocardial cell surface. Thus, by stimulating the production of these antibodies, persistent infection may cause disturbances in heart cell functions, which may lead to some of the complications associated with cChHD. It is worth noting that autoantibodies with agonist-like activities against cardiac receptors were also described in sera from patients suffering from various heart diseases [15–17].

Our previous work revealed that patients with cChHD develop a strong IgG response against the *T. cruzi* ribosomal P2β protein (TcP2β) [18–20]. The main epitope was mapped to the C-terminus of this antigen, a stretch of 13 amino acid residues (peptide R13) that appears to be a marker for severe heart ailments [19,21]. The acidic nature of the R13 amino acid sequence generates anti-R13 antibodies with the ability to cross-react with the acidic epitope of the second extracellular loop of the β1-AR receptor [14]. For this reason, human anti-R13 antibodies are capable of exerting a positive chronotropic effect on cultured cardiomyocytes [14], suggesting that they might be involved in the induction of arrhythmias and/or electrical disorders frequently observed in cChHD. Interestingly, immunization of mice with TcP2β recombinant proteins induced anti-R13 antibodies that presented β1-AR-stimulating activity [22,23] and whose presence was associated with supraventricular tachycardia [22].

DNA immunization, also termed genetic immunization, represents a novel approach to induce both cellular and humoral immune responses. Intramuscular injection of plasmid DNA encoding a foreign gene results in the sustained expression of the foreign gene products and the induction of specific humoral and cellular immune responses within the host. Such results are achieved in spite of the fact that the number of muscle cells transfected is very small, and the amount of protein produced in vivo is usually in the picogram–nanogram range [24]. Given these features, we hypothesized that DNA immunization could be an appropriate model to study the induction of pathogenic anti-P antibodies, since it might reflect better the antigenic presentation that takes place during the chronic phase of *T. cruzi* infection. Indeed, ribosomal proteins are expressed throughout the whole cycle of the parasite, part of the cycle occurs within muscle tissues, and the number of circulating and intracellular parasites is extremely small during the chronic phase of the disease. To test this hypothesis, we compared the anti-TcP2β humoral response elicited after DNA immunization with that induced by recombinant protein-based immunization and experimental and natural *T. cruzi* infections. To our surprise, immunization with naked DNA induced an IgG response that was completely different in its IgG isotype profile, fine epitope recognition and functional activity from that observed during experimental infection and cChHD. Remarkably, DNA immunization failed to induce IgG antibodies directed to the C-terminus of TcP2β, which have been shown to be associated with cardiac alterations.

2. Materials and methods

2.1. Infection of mice

Eight C3H/HeJ mice, 6–8 weeks old, were infected by intraperitoneal injection with 10^4 *T. cruzi* bloodstream parasites of the CL Brener strain. Mice were bled every week from day 14 to day 180 post-infection. Parasitemia was determined with blood from the tail vein by optical microscopy (not shown).

2.2. Human sera and mouse antibodies

Serum samples from individuals with cChHD were obtained as previously described [19]. Monoclonal antibodies (MAbs) 17.2 and 68.3 were produced after immunization with the TcP2β protein and previously characterized in [25]. MAb M16 reacted to the second extracellular loop of the human β1-AR molecule [26].

2.3. Plasmid DNA constructs

The DNA fragment encoding the entire TcP2β protein was amplified by PCR using a genomic clone as template [27]. Forward and reverse primers were designed to incorporate, respectively, *Bam*HI and *Xba*I restriction sites for directional cloning. The forward primer also included the Kozak consensus sequence in order to increase the efficiency of translation [28]. After restriction, the PCR products were inserted into the eukaryotic expression vector pcDNA3 (Invitrogen) to generate the recombinant plasmid pcDNA3/TcP2β. The identity of this construct was further confirmed by DNA sequencing. Plasmid DNA was grown in transformed *Escherichia coli* DH5α. Large-scale purification of the plasmid pcDNA3/TcP2β and pcDNA3 control vector was done by using the Qiagen Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The final pellet was resuspended in saline, and DNA concentration was determined by optical density at 260 nm. Final concentration was adjusted to 1 mg/ml, and DNA was kept at –20 °C until used.

2.4. Immunizations

Before DNA immunization, female BALB/c, 8 weeks old, were anesthetized by intraperitoneal injection of pentobarbital and then injected intramuscularly into each tibialis anterior muscle with 50 μg of pcDNA3/TcP2β or empty pcDNA3 by using a 30-gauge needle. Injections were performed three times at 3-week intervals (days 0, 21, and 42). Serum samples were collected from the tail before the first injection (pre-immune sera, P), and 3 weeks after each immunization (bleeds I–III). For immunization with the TcP2β-derived recombinant protein, BALB/c mice were inoculated intraperitoneally with MBP-TcP2β plus incomplete Freund adjuvant (Sigma Chemical Co., St. Louis, MO), as previously described [22,23]. Control animals received equal amounts of MBP alone.

IgG fractions were prepared from pools of three sera per group of mice. Pools were diluted 1:5 in PBS, and IgG antibodies were precipitated at 40% $(NH_4)_2SO_4$ saturation. Pellets were finally resuspended in PBS at a 1:1 dilution and then dialyzed against the same buffer. For monoclonal antibodies, IgG fractions were prepared from the ascites fluids by $(NH_4)_2SO_4$ precipitation, and final protein concentration was determined by absorbance at 280 nm.

2.5. ELISA

Microtiter plates were coated with 50 µl per well of 4 µg/ml recombinant MBP-TcP2β, 5 µg/ml total *T. cruzi* extract [29] or 40 µg/ml synthetic peptides diluted in 0.1 M carbonate buffer, pH 9.6. A panel of 11 peptides covering the complete sequence of the TcP2β protein was used to define the epitope specificity of the anti-TcP2β antibodies. Synthetic peptides were purchased by Research Genetics (Invitrogen Co., Carlsbad, CA) and contained 15 amino acid residues (except for R13 and 18, which were 13 and 18 amino acids long, respectively), with an overlap of at least five amino acids. Their sequences are shown in Fig. 5A.

After coating, plates were blocked with 4% non-fat milk in PBS and incubated with sera serially diluted in 1% milk-PBS. Bound antibodies were detected with peroxidase-labeled goat anti-mouse or anti-human IgG (1:4000; Sigma Chemical Co., St. Louis, MO) followed by the addition of 3,3′,5,5′-tetramethyl benzidine (TMB) and H_2O_2. The reaction was stopped with 1 N HCl, and the absorbance was read at 450 nm on an automated ELISA microplate reader (Molecular Devices Co., Sunnyvale, CA). OD values were considered positive when they reached a value equal to or more than the mean + S.D. value obtained from controls (i.e., mice immunized with pcDNA3 or MBP, sex- and age-matched uninfected mice, or healthy individuals). For recombinant proteins, all absorbance values represent binding of IgG to the GST-TcP2β protein after subtraction of binding of the same serum to GST. The isotype profile of the anti-TcP2β IgG response in mice and patients was determined using specific antibodies from Caltag (Burlingame, CA) and Oxoid (Hampshire, England), respectively.

2.6. In vitro expression of pcDNA3/TcP2β in COS-7 cells

In vitro expression of pcDNA3/TcP2β was assessed by transient transfection of COS-7 cells. Cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM), and transfections were performed with LipofectAMINE, according to the protocol provided by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). After 24 h in culture, cells were washed twice with PBS, fixed in acetone:methanol (1:1) for 5 min at room temperature and washed four more times with PBS. Cells were then stained for 2 h at 37 °C with a polyclonal anti-TcP2β serum (obtained from a mouse immunized with MBP-TcP2β and diluted 1:2000 in PBS-10% fetal calf serum), washed four times with PBS and incubated for 1 h at 37° C with alkaline phosphatase-labeled goat anti-mouse IgG (Caltag, 1:1000 dilution in PBS-10% fetal calf serum). After four washes with PBS, bound immunocomplexes were detected by incubation with BCIP/NBT (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature. Cells expressing the TcP2β protein were visualized with an inverted microscope.

2.7. Measurement of cAMP levels in COS-7 transfected cells

COS-7 cells were stably transfected with the expression plasmid pcDNA3/β1-AR, encoding the entire human β1-AR molecule. Intracellular levels of cAMP were determined with the Biotrak™ cAMP competitive enzyme immunoassay (EIA) system (Amersham Bioscience, Piscataway, NJ), following the protocol provided by the manufacturers. Cells were incubated with 1:50 final dilution of the IgG preparations, 150 nM of MAbs or PBS for 60 min. Incubations with 1 µM isoproterenol were performed for 5 min. The production of cAMP was expressed as an activation index, i.e., the ratio between the cAMP concentration (pmol/tube) measured after incubation with an IgG preparation and the cAMP concentration measured after incubation with PBS alone. Experiments were performed in duplicate, and values were averaged.

3. Results

3.1. Anti-TcP2β response in infected mice

In order to compare the anti-TcP2β IgG response induced by DNA immunization with that observed during *T. cruzi* infection, it was important to first analyze the kinetics of the anti-TcP2β response in experimentally infected mice. As shown in Fig. 1, infected mice raised an anti-TcP2β IgG response that was detectable 2 months after infection, a month later than the onset of the anti-*T. cruzi* response, and it was maximal 4 months post-infection. The mean antibody titer measured at day 160 post-infection was 1:20,000 (not

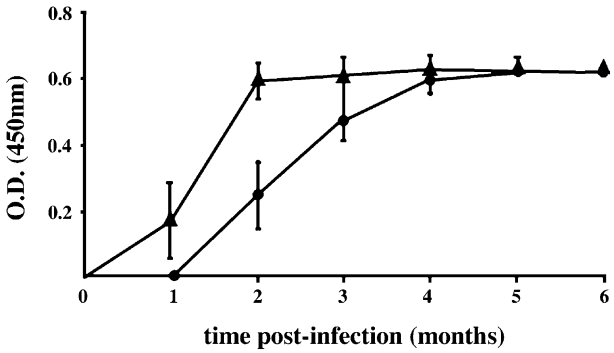

Fig. 1. Anti-TcP2β IgG antibodies in chronically infected mice. Mice infected with *T. cruzi* were bled every month after infection, and sera were tested by ELISA against the GST-TcP2β recombinant protein (circles) or a *T. cruzi* total lysate (triangles). Sera were used at a 1:1000 dilution. Results represent the mean OD value obtained from eight mice ± S.D.

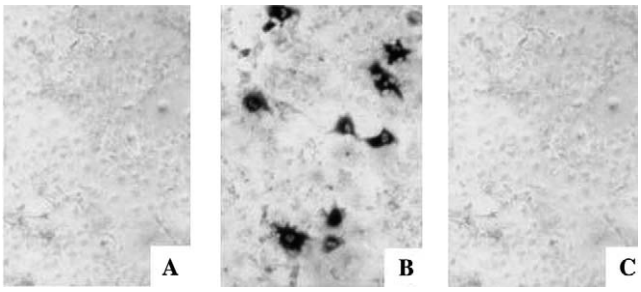

Fig. 2. Cytoplasmic expression of the TcP2β protein in transiently transfected cells. COS-7 cells were transfected with the pcDNA3 vector (A) or the TcP2β-encoding plasmid pcDNA3/TcP2β (B and C). After 48 h, cells were fixed and immunostained with a polyclonal anti-TcP2β serum (A and B) or with a control mouse serum (C).

shown), and these levels persisted along the chronic phase of the experimental disease.

3.2. Expression of TcP2β in transiently transfected COS-7 cells

In order to investigate whether immunization with a plasmid DNA encoding the protein could provoke a pathogenic immune response in mice, the TcP2β gene was subcloned into the pcDNA3 vector. This recombinant plasmid expressed the inserted gene upon transient transfection of COS-7 cells. The cytoplasmic expression of the TcP2β protein was strong as revealed by in situ staining with a polyclonal antiserum (Fig. 2B). In contrast, these cells showed no reaction when incubated with a normal mouse serum (Fig. 2C). No expression was detected in mock cells (not shown) or cells transiently transfected with the control pcDNA3 vector (Fig. 2A). These data clearly show that mammalian cells transfected with pcDNA3/TcP2β present the TcP2β protein in a form that is recognized by anti-TcP2β antibodies.

3.3. Induction of anti-TcP2β antibodies in DNA-immunized mice

BALB/c mice were immunized intramuscularly on days 0, 21, and 42 with the pcDNA3/TcP2β construct or control pcDNA3, and serum samples were taken 3 weeks after each injection. Antibody levels were determined by ELISA on plates coated with the GST-TcP2β fusion protein. In mice immunized with pcDNA3/TcP2β, IgG levels against TcP2β were positive after two doses and maximal after three doses of recombinant plasmid DNA (bleed III, Fig. 3A). Nevertheless, even after three inoculations, antibody levels remained below those observed upon immunization with the recombinant protein MBP-TcP2β (Fig. 3A). As expected, control animals inoculated with pcDNA3, MBP (Fig. 3A) or saline alone (not shown) did not develop antibodies against TcP2β epitopes. For comparison, Fig. 3B shows the anti-TcP2β IgG reactivity of sera from a representative chronically infected mouse and from a patient with Chagas' disease. It is worth noting that all sera from mice immunized with

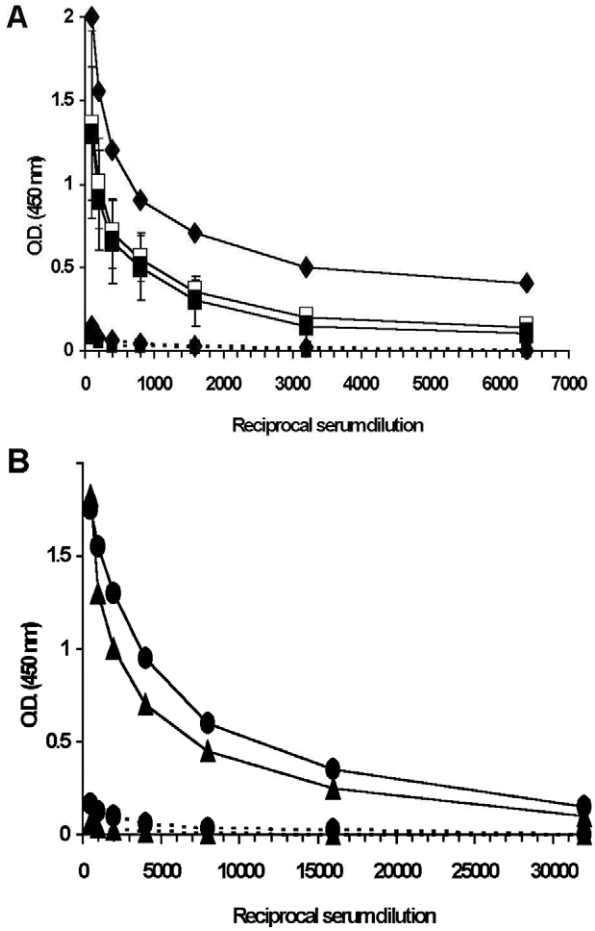

Fig. 3. Antibodies to the TcP2β protein in pcDNA3/TcP2β-immunized mice. (A) Sera from BALB/c mice immunized with plasmid pcDNA3/TcP2β or the recombinant protein MBP-TcP2β were tested by ELISA against the GST-TcP2β fusion protein. Solid lines represent sera from animals immunized with pcDNA3/TcP2β (squares) or MBP-TcP2β (diamonds); dotted lines represent sera from control mice injected with empty pcDNA3 (squares) and MBP alone (diamonds). Open squares correspond to animals immunized with pcDNA3/TcP2β + pcDNA3/GM-CSF. Results represent the mean OD value obtained from eight mice ± S.D. for DNA immunization and the OD values obtained from one representative mouse for the other groups. (B) IgG reactivity of sera from a patient with Chagas' heart disease (solid line, circles) and from a chronically infected mouse (solid line, triangles). Controls include sera from a healthy individual (dotted line, circles) and from an uninfected mouse (dotted line, triangles).

pcDNA3/TcP2β also recognized MBP- or GST-TcP2β by Western blot (data not shown).

Many authors have reported that both humoral and cellular immune responses generated by DNA immunization can be increased by the co-delivery of plasmid DNA expressing immunomodulatory molecules such as GM-CSF (granulocyte-macrophage colony-stimulating factor) [30,31]. Accordingly, we assessed the co-injection of equal amounts of pcDNA3/GM-CSF and pcDNA3/TcP2β, using the same immunization schedule as described above. In our experiments, co-administration with pcDNA3/GM-CSF did not induce any change in the anti-TcP2β IgG levels when compared with those produced by immunization with

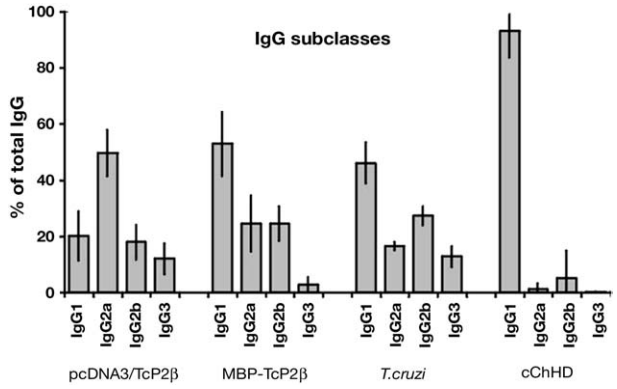

Fig. 4. IgG subclass distribution of the anti-TcP2β antibody response. Serum samples were used at a 1:100 dilution and included those collected from mice immunized with pcDNA3/TcP2β, mice immunized with MBP-TcP2β, chronically infected mice (*T. cruzi*), and patients with Chagas' heart disease (cChHD). Bars indicate the mean (±S.D.) percentages of total anti-TcP2β IgG subclasses ($n = 8$ in pcDNA3/TcP2β, MBP-TcP2β and *T. cruzi* groups and $n = 20$ in cChHD group).

pcDNA3/TcP2β (Fig. 3A). Therefore, further experiments were performed only with the latter construct.

The predominant IgG subclass induced by immunization with pcDNA3/TcP2β was IgG2a, although significant but lower IgG1, IgG2b, and IgG3 levels were also detected. Conversely, the anti-TcP2β IgG antibodies elicited during the *T. cruzi* infection or by immunization with the MBP-TcP2β recombinant protein were mainly of the IgG1 isotype (Fig. 4). No specific IgM antibodies were detected during the DNA immunization scheme (not shown).

3.4. Epitope mapping of the anti-TcP2β antibodies

We analyzed the fine specificity of the anti-TcP2β antibodies induced by DNA immunization. Serum samples (bleed III) were tested against a panel of 11 overlapping peptides expanding the whole protein (Fig. 5A). Each serum reacted at least to one peptide when assayed by ELISA. As shown in Fig. 5B, DNA immunization elicited antibodies predominantly directed to the internal peptides 1, 7, and/or 8. In contrast, IgG antibodies from chronically infected mice or patients with cChHD exclusively reacted with the C-terminal R13/R18 peptides. Interestingly, mice immunized with the MBP-TcP2β fusion protein produced antibodies recognizing both C-terminal R13/R18 peptides and internal peptides (peptides 3, 4, 7, and/or 8, Fig. 5B). As reference, MAbs 17.2 and 68.3 recognized peptides R13 and 7, respectively (Fig. 5C).

3.5. Functional activity of the antibodies elicited by immunization with pcDNA3/TcP2β

The functional activity of the anti-TcP2β IgGs was assessed by measuring the cAMP production of COS-7 cells stably transfected with the β1-AR gene. Incubation with IgGs from mice immunized with pcDNA3/TcP2β did not modify the cAMP basal levels, as was observed for IgGs

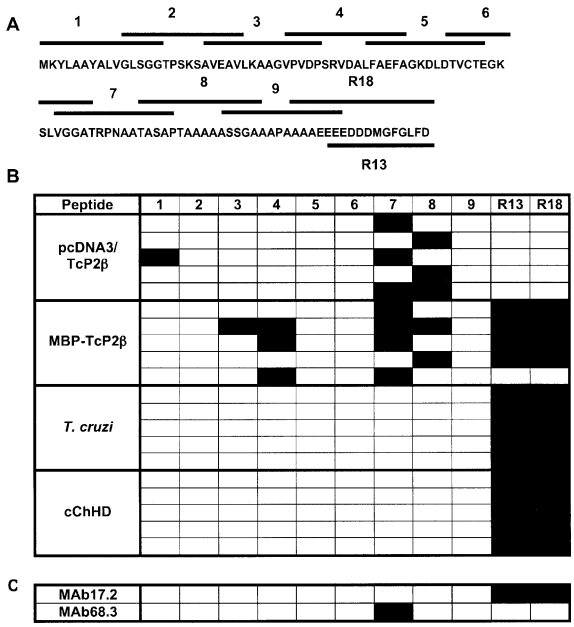

Fig. 5. Fine epitope mapping of the anti-TcP2β humoral response. Sera were tested by ELISA against a panel of 11 overlapping peptides expanding the complete TcP2β protein. (A) Amino acid sequences of the synthetic peptides used in the experiment. (B) Reactivity of different groups of sera to peptides indicated in (A). Serum samples were collected from mice immunized with pcDNA3/TcP2β or MBP-TcP2β, or chronically infected (*T. cruzi*), and from patients with cChHD. Sera from immunized mice were tested at a 1:100 dilution, while sera from infected mice and patients were diluted 1:1000. Five sera in each group are shown in the figure as representative examples. (C) Reactivity of MAbs17.2 and 68.3. Open and closed boxes represent negative and positive reactions, respectively.

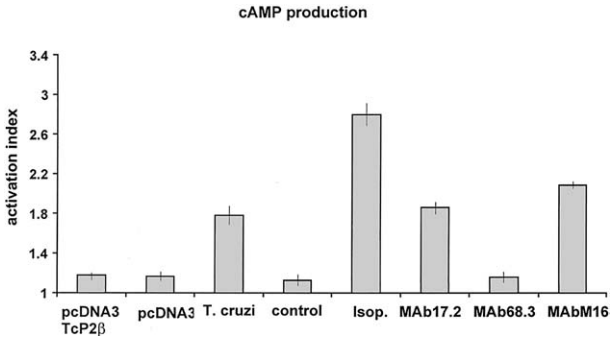

Fig. 6. cAMP production in COS-7 cells stably expressing the β1-AR molecule. Transfected cells were incubated with IgG antibodies obtained from mice immunized with pcDNA3/TcP2β or empty pDNA3, and from mice chronically infected with *T. cruzi* or non-infected control mice. cAMP production induced by incubation with isoproterenol (Isop.), and MAbs 17.2, 68.3 and M16 was measured as control. Bars indicate the mean (±S.D.) cAMP concentrations expressed as activation indexes (see Section 2) and obtained from two independent assays.

from mice immunized with pcDNA3 alone, non-infected mice and MAb 68.3 (Fig. 6). Conversely, IgGs from chronically infected mice significantly increased the cAMP levels of COS-7 transfected cells. Similar results were obtained after incubation with the β1-AR agonist isoproterenol and MAbs 17.2 and M16 (Fig. 6). It is important to recall here

that MAb M16 is directed to the second extracellular loop of the β1-AR molecule [26].

4. Discussion

DNA immunization represents an interesting approach to elicit specific immune responses against a given antigen. Although this technology has been extensively used as a vehicle for vaccine and immunotherapeutic development, we chose to use it in this study to induce pathogenic antibodies capable of stimulating cardiac receptors. In intramuscular DNA immunization, foreign antigens are expressed primarily within muscle cells, which are weak antigen-presenting cells (APCs) for inducing immunity. However, DNA immunization is likely to involve professional APCs that may be directly transfected with the plasmid DNA or may also capture antigens synthesized by myocytes (rev. in [24]). One of the distinct aspects of the T cell response induced by this type of immunization is the predominance of IgG2a antibodies and cytokines such as IL-2 and IFNγ, which are characteristic of a type 1 phenotype (Th1). In contrast, injection of purified proteins together with adjuvants usually elicits a mixed T helper phenotype or even stimulates a predominant Th2 response with IgG1 antibodies and cytokines such as IL-4 [32,33]. Accordingly, the results presented herein show that intramuscular delivery of the pcDNA3/TcP2β DNA construct mainly raised IgG2a, whereas intraperitoneal injection with the recombinant protein MBP-TcP2β predominantly raised IgG1.

Epitope mapping of the anti-TcP2β humoral response revealed that DNA-based immunization elicits antibodies that exclusively recognize internal peptides on TcP2β whereas immunization with the corresponding recombinant protein induces antibodies that recognize both internal and C-terminal peptides of the protein. This difference could be due to the processing pathway, the amount of antigen, and the presentation of the antigenic determinants to the immune system. First, the endogenously synthesized antigen after DNA injection is presented by MHC class I, while the uptake of the soluble recombinant protein by specialized APCs allows presentation by MHC class II (rev. in [34,35]). Secondly, DNA-based immunization results in the expression of extremely small quantities of the foreign protein when compared with those delivered by recombinant protein-based immunization [24]. Lastly, different types of APCs may be recruited, depending on the immunization mode. In fact, Doe et al. [36] suggested that APCs of hematopoietic origin appear to be involved in the induction of immune responses following intramuscular injection of plasmid DNA [36].

Our results also confirmed that the anti-TcP2β antibodies elicited during experimental *T. cruzi* chronic infection and cChHD are exclusively directed to the C-terminus of the TcP2β antigen (R13/R18 peptides). The different patterns of recognition between infection and immunization may be explained by polyclonal activation occurring during *T. cruzi* infection (rev. in [37]). In fact, this phenomenon has been associated with an increase in natural and multi-specific antibodies, leading to maturation of anti-self antibodies [29,38]. Alternatively, the lack of recognition of internal peptides may be due to the immunosupression that has been shown to take place during the acute phase of *T. cruzi* infection [39] and/or to impaired APC function caused by a defective MHC class II presentation by *T. cruzi*-infected macrophages [40].

This study also stresses the tight correlation that exists between the presence of antibodies directed to C-terminal peptides of TcP2β and the functional activity against cardiac receptors. Indeed, we have previously reported that mice immunized with MBP-TcP2β develop anti-P antibodies that recognize the C-terminal peptide R13 and exert a chronotropic effect on spontaneously beating neonatal myocytes [22,23]. This functional activity has also been measured using anti-R13 antibodies obtained from patients with cChHD [14]. Here, the existence of anti-receptor antibodies was investigated by measuring the cAMP levels produced by COS-7 cells stably transfected with the β1-AR. The intracellular levels of cAMP in transfected cells increased in the presence of anti-R13 IgG preparations (i.e., IgGs from chronically infected mice) but not IgG preparations lacking anti-R13 antibodies (i.e., IgGs from mice immunized with pcDNA3-TcP2β). This finding is in agreement with the fact that only MAb17.2 but not MAb68.3 increased the levels of cAMP in COS-7 cells. Interestingly, MAb17.2 reacts with the C-terminal peptide R13, while MAb68.3 is directed to the internal peptide 7 ([25] and Fig. 4). IgG antibodies from mice immunized with MBP-TcP2β also increased the cAMP levels of β1-AR transfected cells, as previously described by López Bergami et al. [22]. In accordance with results discussed above, mice immunized with pcDNA3/TcP2β presented no changes in their electrocardiographic tracings (data not shown), in contrast with mice immunized with the recombinant protein MBP-TcP2β that developed anti-R13 antibodies [22,23].

Although DNA-based immunization cannot mimic the anti-TcP2β humoral response developed during *T. cruzi* infection, our data indicate that the recognition of antigenic determinants can be manipulated to obtain an anti-TcP2β antibody response that is totally different from that observed in cChHD. In particular, immunization with the plasmid pcDNA3/TcP2β induces IgG antibodies directed to internal regions of the protein, avoiding the production of pathogenic antibodies. Recently, it has been shown that immunization with pcDNA3/TcP2β induces MHC class I-restricted cytotoxic T lymphocytes (CTLs) specific for the N-terminal peptide of the TcP2β molecule [41]. Remarkably, these immunized mice presented a lower degree of early parasitemia after parasite challenge. Given these features, TcP2β epitopes, except those of the C-terminus, may well be considered putative components of a future multigene DNA vaccine against *T. cruzi*.

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