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Elicitation of specific, Th1-biased immune response precludes skeletal muscle damage in cruzipain-vaccinated mice

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Abstract

Cruzipain (Cz), the major cystein proteinase of *Trypanosoma cruzi*, is able to induce protective immunity against parasite challenge. However, some concern has arisen regarding its potential to elicit pathogenic autoimmune reactivity. To determine whether the adverse myopathic effects of Cz-based immunization could be prevented, we evaluated the co-administration of Cz with different adjuvants. Mice were immunized with Cz adjuvantized by alum (Cz+alum), oligodeoxynucleotides containing CpG motifs (Cz+ODN-CpG) or Freund's preparation (Cz+CFA). Cz triggered a vigorous specific humoral response, irrespective of the adjuvant used. Alum mainly drove response towards Th2 phenotype, characterized by specific IgG1 antibodies and IL-10 induction, whereas Cz+ODN-CpG mice exhibited Th1-dominant immunity, with antibodies of the IgG2a isotype and enhanced IFN-γ production. Histological examination of cardiac tissue demonstrated lesions in Cz+CFA but not in Cz+alum nor Cz+ODN-CpG immunized animals, suggesting that CFA is critical for Cz-mediated injury. Analysis of skeletal muscle revealed that mice receiving Cz+CFA exhibited disrupted and hyalinized myofibers, whereas [Cz+alum]-immunized animals showed hyalinization, architecture modifications and small inflammatory foci. Conversely, no abnormalities were observed in the striated muscle from the Cz+ODN-CpG group. Hence, generation of specific immune response skewed towards Th1, as that recorded for the ODN-CpG adjuvant, may preclude triggering of Cz-mediated muscle tissue damage.

Keywords: Chagas' disease; Skeletal muscle; Cruzipain; Immune response; *Trypanosoma cruzi*

Introduction

Chagas' disease (American Trypanosomiasis) is a parasitic infection caused by the protozoan *Trypanosoma cruzi* and transmitted by a reduviid insect that affects 16–18 million people in Central and South America. In these regions, 70% of the population is at risk of contracting the disease (TDR, 1999). Chagas' disease is characterized by an acute phase, with high parasitemia generally self-limited, followed by an indeterminate stage that can last for years without signs or symptoms. Between 20 and 30% of patients progress to a chronic phase, along which different types of heart pathology may appear, such as mild arrhythmia, right or left branch block and severe cardiomyopathies which can cause death. Disorders of the esophagus and/or colon (megaviscera) may also be present in other chronic patients. Since chemotherapy of Chagas' disease has limited efficacy and is not innocuous, it is necessary to develop vaccines able to induce a protective immunity against *T. cruzi* infection, revert an ongoing infectious process and/or prevent the pathology caused by the parasite.

Different mechanisms have been proposed to explain the immunopathology observed at the chronic stage of Chagas' disease, e.g. serum antibodies against homologous antigens

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between *T. cruzi* and mammals, such as some ribosomal proteins (Skeiky et al., 1992), laminin (Szarfman et al., 1982; Silva-Barbosa and Savino, 2000), as well as certain components of the nervous system (Wood et al., 1982; Van Voorhis and Eisen, 1989) and skeletal muscle (Zwirner et al., 1994; Guinazu et al., 2004; Iwai et al., 2005). Several *T. cruzi* antigens have been proposed to elicit an autoimmune response in infected mammals. The parasite ribosomal protein R13 cross-reacts with a 23-kDa host ribosomal protein, β1 adrenergic receptor and M2 muscarinic receptor (Lopez Bergami et al., 1997; Mahler et al., 2004), while B13 and cruzipain (Cz) may cross-react with myosin heavy chain (Giordanengo et al., 2000a,b; Iwai et al., 2005). Further knowledge of parasite antigens inducing either pathogenic or protective responses will be helpful in understanding Chagas' pathogenesis and in the design of safe and effective vaccine approaches for this parasitosis.

In a previous work, we showed that Cz, the major *T. cruzi* cystein proteinase, is a relevant candidate for a *T. cruzi* protective vaccine (Frank et al., 2003). Cz is expressed in all strains and developmental forms, it accumulates in lysosomes, is secreted in the flagellar pocket and it is also present at surface level, being able to hydrolyse IgG (Berasain et al., 2003). Moreover, specific enzyme inhibitors interfere with cell invasion and *T. cruzi* replication (Cazzulo et al., 2001). Cz acting as immunogen in a T helper type 1 (Th1) context is able to diminish parasitemia and increase survival rates in acutely infected mice. Protection against *T. cruzi* was achieved using both recombinant and natural (parasite-derived) Cz through different strategies aimed to drive Th1-biased immunity, such as co-immunization with recombinant IL-12 and anti-IL-4 monoclonal antibodies (mAb) (Schnapp et al., 2002) or, more recently, co-administration with synthetic oligodeoxynucleotides containing CpG motifs (ODN-CpG; Frank et al., 2003). On the other hand, Cz by itself predominantly induced a Th2 cytokine profile (Giordanengo et al., 2002 and Stempin et al., 2002), generating both antibody- and cell-mediated autoreactivity against myosin and subsequently leading to functional and structural alterations at the heart and skeletal muscle (Giordanengo et al., 2000a,b). Consequently, the same antigen used for immunization in different experimental models may be effective in preventing Chagas' disease or responsible for pathogenesis.

One factor decisively contributing to the final outcome of Cz-based immunization is the delivery of an adjuvant along with the parasite immunogen. Experimental adjuvants are known to differentially favor the development of Th1 or Th2 immune responses to defined antigens (Vogel, 2000). For instance, ODN-CpG is capable of driving the response towards Th1 whereas alum has long been characterized as a Th2-promoting adjuvant. To gain insight into the immunopathogenic and immunoprotective mechanisms involved in the response raised by Cz, we analysed the effects of this key *T. cruzi* antigen when co-administered with different adjuvants into C3H/HeN mice. This murine strain was chosen for our studies based on the feature that it does not exhibit a predominantly defined Th basal response, is susceptible to experimental autoimmune disease (Tokunaga et al., 1993; Sakamoto et al., 1998) and, more importantly, develops a protective response to *T. cruzi* upon immunization with Cz (Frank et al., 2003). Among the different adjuvants tested, we included alum, licensed for human use; ODN-CpG, currently evaluated in clinical trials; and complete Freund's preparation (CFA), the standard adjuvant in animal models. Our results indicate that tissue injury generated upon Cz immunization relies on the type of T helper response triggered which, in turn, is partially dependent on the kind of adjuvant used to enhance vaccine performance.

Materials and methods

Cz purification

T. cruzi epimastigotes, RA strain, grown in biphasic medium (Chiari and Camargo, 1984) were harvested during exponential growth, resuspended in 0.25 M sucrose and 5 mM KCl containing protease inhibitors (2 μM PMSF, 5 μM leupeptin, 5 μM pepstatin and 5 μM E-64; Sigma, St. Louis, MO) and broken by cycles of freezing and thawing. After centrifugation at 105,000 ×g, the obtained supernatant was used as an antigen source. Purified mAb 163B6 was fixed to Sepharose 4B-BrCN and used as immunosorbent to purified Cz as described in Frank et al. (2003). Eluted antigen was dialyzed against PBS and conserved at −70 °C until use.

Myosin purification

Cardiac myosin H chains were purified according to Shiverick et al. (1975). In brief, hearts from C3H/HeN mice were removed, washed, minced and finally homogenized in 10 vol of ice-cold KCl buffered solution, pH 6.8 (0.3 M KCl, 0.15 M K_2HPO_4, 10 mM $Na_4P_2O_7$, 1 mM $MgCl_2$). After centrifugation for 1 h at 140,000 ×g, the supernatant was precipitated in 20 vol of water for 18 h at 4 °C. The precipitate was separated by centrifugation for 30 min at 12,000 ×g and suspended in ice-cold imidazole buffer, pH 6.8 (0.5 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 5 mM Na_2ATP, 2 mM dithiothreitol), and further centrifuged for 30 min at 43,000 ×g to remove actin. Myosin was precipitated again with 8 vol of water for 18 h at 4 °C. Residual actin was removed following the above mentioned steps.

Protein concentrations were determined by Bradford and purity analysed by SDS-PAGE. Before inoculation into mice, or for *in vitro* stimulations, antigens were filtered throughout a 0.2 μm pore membrane.

Immunizations

Studies were carried out in inbred, female 6–8 week-old C3H/HeN mice from the University of Buenos Aires, Argentina, with 10 animals in each experimental group maintained under standard conditions. Mice were immunized, and boosted one week later, by bilateral injection in the quadriceps with 10 μg of Cz added to 100 μg of alum (Al_2O_3, Alhydrogel, Superfos Biosector, Vedbaek, Denmark) or ODN-CpG 1826 (Oligos Etc., Wilsonville, OR). The ODN-CpG was synthesized under GMP conditions, with a nuclease-resistant phosphorothioate backbone and its sequence was 5′-TCC ATG ACG TTC CTG ACG TT-3′. Additionally, other groups of mice were immunized with 10 μg Cz or myosin plus 100 μl of CFA. Control groups received either PBS, Cz, alum, ODN-CpG or CFA alone. Blood was collected from the tail vein at different time points and the sera analysed for the presence of specific antibodies. Mice were killed by cervical dislocation two weeks after the last immunization.

Antibody titers and IgG isotype determination

An indirect ELISA for antibody detection was used as described by Voller et al. (1988). Plates (Nunc, Roskilde, Denmark) were sensitized with 0.1 μg/well of Cz. Peroxidase-conjugated goat immunoglobulins to mouse IgG (Sigma), or biotinylated rat mAbs to mouse IgG1 or IgG2a subclasses (Pharmingen, San Diego, CA) were used as secondary antibody, followed by streptavidin-