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Humoral and cellular immune responses in BALB/c and C57BL/6 mice immunized with cytoplasmic (CRA) and flagellar (FRA) recombinant repetitive antigens, in acute experimental *Trypanosoma cruzi* infection

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Abstract In previous studies, cytoplasmic repetitive antigen (CRA) and flagellar repetitive antigen (FRA) proteins induced specific humoral and cellular immune responses in susceptible and resistant mice in the absence of *Trypanosoma cruzi* infection with a significant induction of the Interferon-gamma (IFN-γ) production in those animals. In this follow-up paper, the immunostimulatory and protective effects of these proteins were evaluated by immunizing with CRA or FRA antigens, BALB/c and C57BL/6 mice and challenging with a *T. cruzi* (Y strain). Both proteins induced humoral response with high levels of IgG isotypes as well as cellular immunity with high levels of IFN-γ when compared to controls. However, the lymphocyte proliferative response was minimal. The survival rate at 30 days post-infection was significant in CRA (60%) or FRA (50%) - immunized BALB/c mice and CRA (83.3%) - immunized C57BL/6 mice. Taken as a whole these findings indicate that CRA and FRA are immunogenic and potentially important for protective immunity.

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Introduction

Chagas' disease, an infection caused by the protozoan parasite *Trypanosoma cruzi*, affects several million people in the American hemisphere (WHO 2002). Under natural conditions, *T. cruzi* is transmitted to humans by a haematophagous reduviid insect vector, which deposits its infective feces or urine on the skin of the host at the time of biting, this being the most significant route of transmission in endemic areas. However, *T. cruzi* may be transmitted to man by a number of alternative mechanisms (blood transfusion, organ transplantation, accidental laboratory infection, oral or congenital transmission (Gomes 1997). However, blood transfusion is the dominant means of transmission in non-endemic areas.

T. cruzi infections are associated with immunological and immunopathological reactions that may result from non-specific polyclonal activation (Kierszenbaum 1981; Minoprio et al. 1986) or supressive effects (Ramos et al. 1979; Kierszenbaum et al. 1980; Abrahamsohn and Coffman 1995). Experimental infection with *T. cruzi* induces cytokine production involved in the regulation of immune responses. Interferon-gamma (IFN-γ) has been demonstrated to be a protective cytokine against *T. cruzi*. Macrophages activated by IFN-γ release reactive oxygen metabolites and nitric oxide (NO) (Vespa et al. 1994). In addition, the immune control of *T. cruzi* infection involves the participation of $CD4^+$ and $CD8^+$ T cells (Sher and Coffman 1992). Studies with CD4 and CD8 KO mice that are highly susceptible to the parasite

showed the importance of both T-cell populations during naturally acquired immune responses to *T. cruzi* infections (Rottenberg et al. 1993).

Despite the importance of cellular immune responses, anti-parasite humoral responses also play a major role in resistance to *T. cruzi* infection (Brener and Krettli 1990). The major antibody isotypes involved in protection are IgG1 and IgG2 (Araújo et al. 1984; Takehara et al. 1981; Brodskyn et al. 1989). These findings showed the importance of the humoral and cellular immune responses in the control of parasitemia and mortality during *T. cruzi* infection.

Several recombinant antigens have been used to evaluate protective immune responses against *T. cruzi* infection (Taibi et al. 1995; Santori et al. 1996; Pereira-Chioccola et al. 1999; Millar et al. 2000; Schnapp et al. 2002). We have previously demonstrated the immunogenicity of cytoplasmic repetitive antigen (CRA) and flagellar repetitive antigen (FRA) recombinant proteins of *T. cruzi* by evaluating the humoral and cellular responses in uninfected susceptible (BALB/c) and resistant (C57BL/6) mice strains immunized with these proteins (Pereira et al. 2003a, 2003b, 2004). These antigens were able to elicit the production of specific IgG immunoglobulins. CRA induced predominantly the production of IgG1 and IgG3 isotypes in BALB/c and C57BL/6 mice, while FRA induced antibodies of the IgG1 isotype in BALB/c mice (Pereira et al. 2003a, 2003b). Cellular lymphoproliferative response was also observed in both mouse strains. Moreover, spleen cell cultures mainly from CRA-immunized C57BL/6 and FRA-immunized BALB/c mice produced high levels of IFN-γ, indicating that these antigens brought about a significant up-regulation of the Th1-type immune response (Pereira et al. 2004).

In the present paper, the humoral and cellular responses in mice immunized with CRA or FRA recombinant antigens and challenged with the Y strain of *T. cruzi* were evaluated to detect a protective effect of theses antigens against host infection or spread of parasite infection within the host.

Materials and methods

Mice

Six- to eight-week-old BALB/c and C57BL/6 male mice from the Animal Breeding Center-Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, were used. The guidelines of the Ethical Committee for the use of experimental animals of the Fundação Oswaldo Cruz/FIOCRUZ (Ministry of Health, Brazil) were followed.

Antigens

CRA and FRA antigens were obtained as described by Krieger et al. (1992). Briefly, the genes encoding CRA and FRA were cloned and their expression induced using isopropyl-β-D-thiogalactoside (IPTG). After centrifugation, the proteins were purified by nickel affinity chromatography according to the supplier's directions (Qiagen).

Immunization and infection

Fifty mice of each strain (BALB/c and C57BL/6) were injected three times by subcutaneous route at 20-day intervals with 20 µg and 12 µg of CRA or FRA, respectively (equimolar doses), according to Pereira et al. (2004). Mice were infected intraperitoneally with 10^4 blood-form trypomastigotes of the Y strain of *T. cruzi*, 3 weeks after the last immunization, as described by Gomes et al. (1999). Parasitemia was measured using Brener's technique (1962), from the fifth to the thirtieth day post-infection (d.p.i.). Dead mice were removed daily. The humoral and cellular immune responses were evaluated at 7, 14 and 30 d.p.i.

ELISA assay for measurement of murine IgG isotype serum levels

ELISA was performed as described by Pereira et al. (2003a). Briefly, microtiter plates (Nunc-Immuno Plates, MaxiSorp, 96 wells, Nalge Nunc International Corporation) were coated with 1 µg/ml of CRA or FRA (100 µl/well) diluted in 0.05 M Na_2CO_3buffer, pH 9.6 and incubated overnight at 4°C. Mice serum (100 µl) diluted 1:100 was added and incubated overnight. After washing, the plates were incubated with peroxidase-labelled rabbit antibodies to mouse immunoglobulins isotypes (anti-IgG1, anti-IgG2a, anti-IgG2b, and anti-IgG3) and the reaction brought on by addition of orthophenyldiamine-OPD and H_2O_2. The optical density (OD) was measured at 490 nm on a BioRad 3550 plate reader.

Proliferation assay

Spleens and inguinal lymph nodes were removed aseptically. Cell suspensions from CRA- or FRA-immunized and control mice (pools of three mice per group) were prepared and cultured in a 96-well plate at a density of $4x10^5$ cell/well) in an RPMI-1640 supplemented with 10% of fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) for 72h at 37°C in 5% CO_2. The cultures were stimulated in the absence or the presence of CRA (20 µg/ml) or FRA (12 µg/ml) antigens, and with concanavalin A (Con A; 2.5 µg/ml). Eighteen hours before harvesting, the plates were pulsed with 0.5 μCi/well with [^{3} H] TdR (Amersham Pharmacia, Little Chalfont, UK). Isotope incorporation was determined by liquid scintillation counting and the results were expressed as the arithmetic mean counts per minute (cpm) of triplicate sample $\pm$ standard deviation (SD).

Measurement of cytokine levels in splenocyte supernatants

Spleen cells (10^7 cells/well) were stimulated for 24 h with the same antigens and concentration used in proliferative assays. The levels of the cytokines IL-4, IL-10, TNF-α and IFN-γ were measured using specific two-site ELISA according to the manufacturers suggested protocols. The antibody pairs used for the detection of IL-4 (detection limit 31.3 pg/ml), IL-10 (detection limit 78 pg/ml), TNF-α (detection limit 6.25 ng/ml) and IFN-γ (detection limit 156 pg/ml) were purchased from R&D Systems. All samples were assayed in duplicate and read at 405 nm on an automatic Bio-Rad 3550 ELISA plate reader. The concentrations were determined with reference to a standard curve.

Morphological study

Three infected mice from each group sacrificed after 7, 14 and 30 d.p.i., as well as those which died spontaneously, were autopsied. Samples of heart and spleen were fixed in 10% neutral formalin, embedded in paraffin and sectioned at 5μm. Sections were routinely stained with haematoxylin and eosin.

Statistical analysis

Data were analyzed using the following methods: Mann–Whitney *U*, Kaplan–Meier and Bonferroni tests were used to detect differences between the groups. SPSS v.8—Statistical Package for Social Sciences Incorporation, US and EXCEL (Microsoft, US) were the programs used. Values of $P < 0.05$ were taken to be significant.

Results

Parasitemia and mice survival rates

The mean of parasites/50 microscopical fields of mice blood and survival rates are shown in Table 1. Parasitemia was lower in challenged C57BL/6 mice immunized with CRA and FRA recombinant antigens than in BALB/c mice. In both strains, the peak of parasitemia was detected at 8 d.p.i., in a similar way as in control mice. The course of *T. cruzi* infection was equivalent, in all the groups, but the survival rates showed significant differences ($P < 0.05$) (Fig. 1). At 30 d.p.i. they were higher, in CRA (60%) or FRA (50%) – immunized BALB/c mice and in CRA (83.3%) – immunized C57BL/6 mice, when compared to those seen in control mice. All infected BALB/c mice that were not previously immunized with the recombinant antigens died after challenge. However, 16.6% of the control C57BL/6 mice survived.

Production of IgG isotypes

Trypanosoma cruzi infection in BALB/c and C57BL/6 immunized mice increased the levels of IgG isotypes. At 7 d.p.i., the levels of all IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) in CRA-immunized BALB/c and C57BL/6 mice were higher ($P < 0.05$) when compared to the control mice (Fig. 2a, 3a). In CRA or FRA-immunized BALB/c mice results were not significant even at 30 d.p.i. (Fig. 2b, d). Figure 3b also shows the ELISA used for detection of antibodies against CRA in C57BL/6, results after 30 d.p.i. being similar to previous time points. All IgG subclasses of FRA-immunized BALB/c mice increased significantly ($P < 0.05$) at 7 d.p.i. (Fig. 2c). In contrast, in infected FRA-immunized C57BL/6 only the IgG2b isotype produced increased

Table 1 Parasitemia and survival rates in mice acutely infected with the Y strain of *T. cruzi* 30 days post-infection

Variables	BALB/c			C57BL/6		
	Control	CRA	FRA	Control	CRA	FRA
Parasitemia[a]						
5 d.p.i.	8.25 ± 2.2	2.8 ± 1.2	7.5 ± 2.5	1.8 ± 0.6	0.3 ± 0.2	1.3 ± 1.3
6 d.p.i.	2.75 ± 0.8	1.6 ± 0.2	2.75 ± 0.9	2.2 ± 0.9	0.7 ± 0.3	1.2 ± 1.2
7 d.p.i.	90 ± 23	41.8[b] ± 9.8	93.5 ± 29.9	9.5 ± 2.3	2.2[b,c] ± 1.2	10.5 ± 10.5
8 d.p.i.	152.7 ± 53	54.4 ± 10.7	84.5 ± 22.9	2.5 ± 9.8	17 ± 2.9	24.3 ± 24.3
9 d.p.i.	14 ± 3.4	18.6[c] ± 6.5	5 ± 1.5	2.5 ± 1	2.2 ± 0.4	3.5 ± 3.5
14 d.p.i.	44.5 ± 7	22.2 ± 6	24 ± 10.2	3.8 ± 1.2	4.2 ± 1.5	8.8 ± 8.7
17 d.p.i.	[d]	18.5 ± 4.9	30 ± 10.4	9.4 ± 2.7	3.8 ± 2.7	13.8 ± 1.62
21 d.p.i.	[d]	32 ± 14.3	15.5 ± 3.5	6.8 ± 2.9	4 ± 2.9	6 ± 1
30 d.p.i.	[d]	17 ± 10	14 ± 6	10 ± 0	2 ± 0	4 ± 1
Survival[e] (%)	0	60	50	16.6	83.3	16.6

d.p.i. days post-infection

[a]values represent mean of parasites/50 fields of five mice group ± SE (standard error)

[b]$p < 0.05$ CRA or FRA-immunized compared with the control

[c]$p < 0.05$ CRA-immunized compared with FRA-immunized

[d]dead mice

[e]the survival rate represents the percentage of mice died at 30 d.p.i.

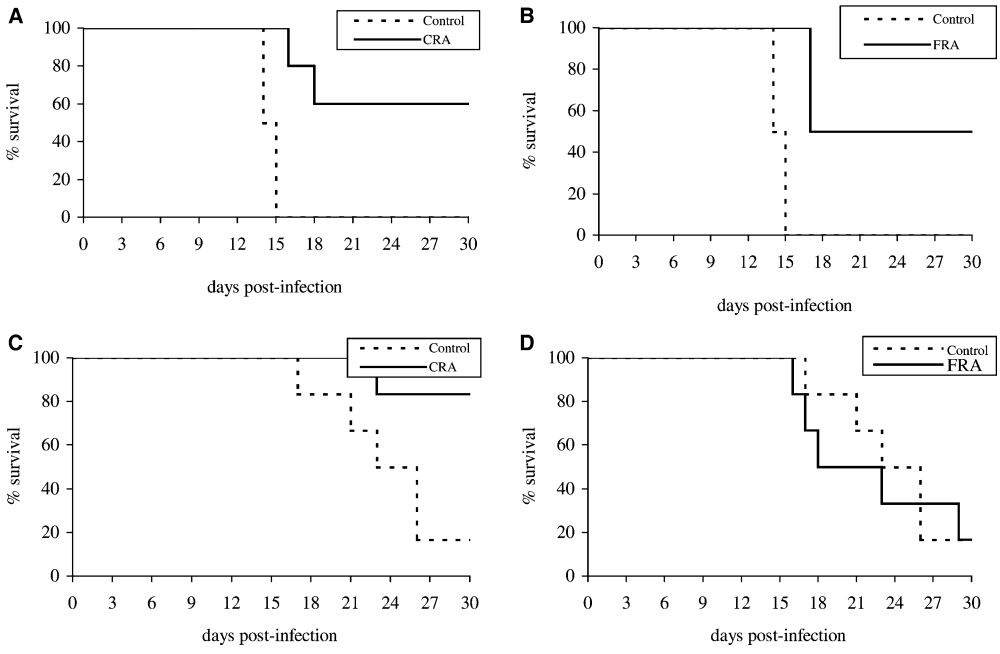


Fig. 1 Survival rate of CRA- (**a**) and FRA- (**b**) immunized BALB/c and CRA (**c**) and FRA (**d**) immunized C57BL/6 during infection with strain Y *T. cruzi*. Groups of five animals were analysed

(Fig. 3c). Significant differences did not occur between the IgG isotypes at 30 d.p.i. (Fig. 3d).

Cytokines production

Supernatants obtained from spleen cells cultures of both the mouse strains showed the highest levels of IFN-γ at 30 d.p.i., as compared to controls (Fig. 4). In BALB/c mice they increased significantly after immunization with both the recombinant antigens (CRA and FRA) (Fig. 4a, b). In C57BL/6 mice, however, significant amounts of IFN-γ were produced 30 d.p.i. upon FRA immunization, but the CRA antigen did not induce the same result (Fig. 4d, c). Although IL-4, IL-10 and TNF-α were detected in the supernatant of cultures, their levels were not different from those detected in controls.

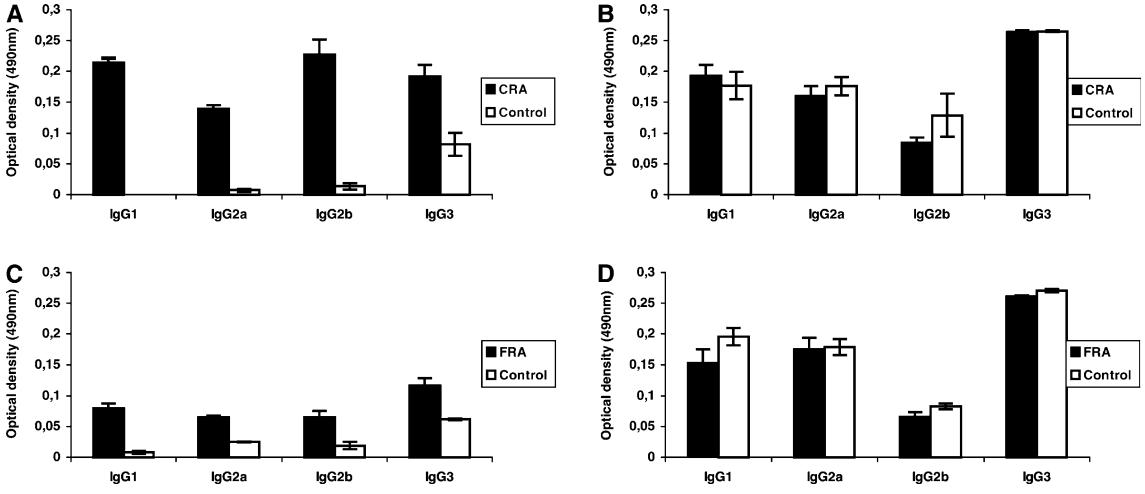
Lymphocyte proliferation assays

Cellular proliferation was minimal or undetectable during the experimental infection. Spleen cells from infected BALB/c and C57BL/6 mice did not proliferate when stimulated in vitro with CRA, FRA, or Con A, indicating that the stimulus responsiveness of these cells was suppressed. Lymph node cells of both strains of mice responded only to Con A at 7 d.p.i.. After 14 d.p.i., the proliferation of lymph node cells was null.

Morphological study

At 7 d.p.i, no heart lesions were seen in CRA- or FRA-immunized BALB/c and C57BL/6 mice. Hypercellular-

Fig. 2 Subclasses of antigen specific immunoglobulins in the sera of CRA (**a**, 7 d.p.i., **b**, 30 d.p.i) and FRA (**c**, 7 d.p.i., **d**, 30 d.p.i.) immunized BALB/c infected mice. The results are expressed as the average of 5 mice ± SD



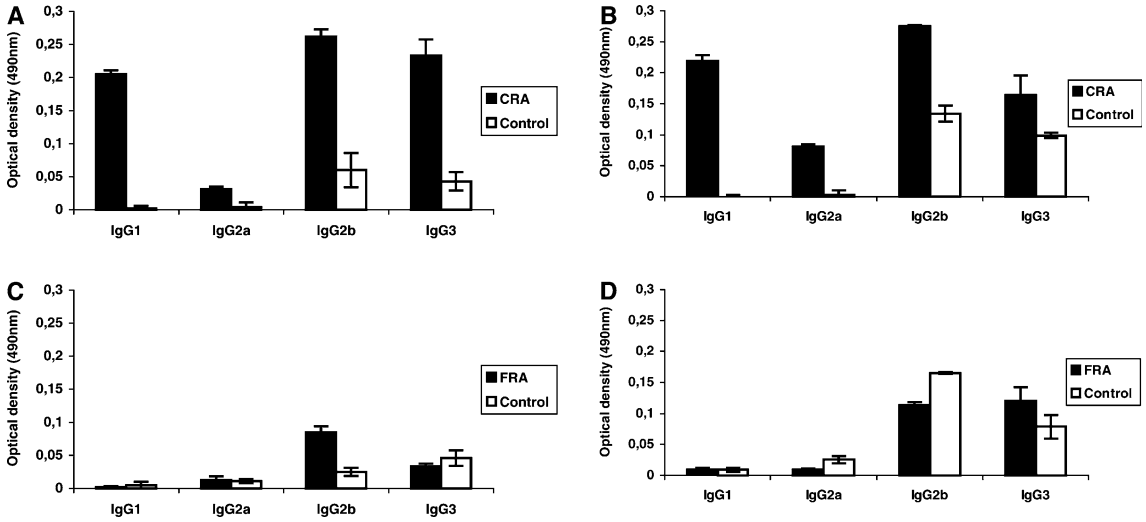
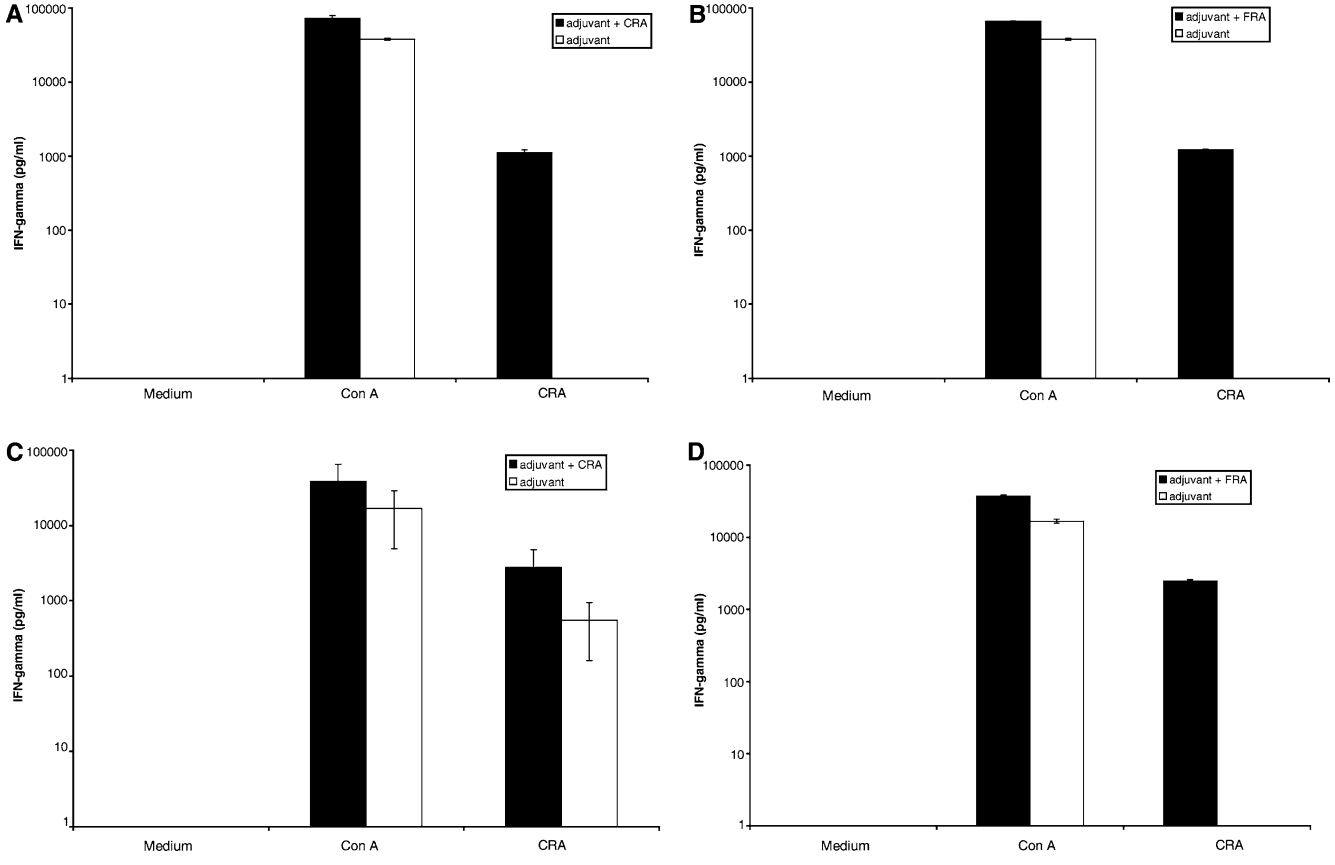


Fig. 3 Subclasses of antigen specific immunoglobulins in the sera of CRA (**a**, 7 d.p.i., **b**, 30 d.p.i) and FRA (**c**, 7 d.p.i., **d**, 30 d.p.i.) immunized C57BL/6 infected mice. The results are expressed as the average of 5 mice ± SD

Fig. 4 IFN-γ produced by T cells from CRA (**a**) or FRA (**b**) -immunized infected BALB/c mice and CRA (**c**) or FRA (**d**) -immunized infected C57BL/6 mice. Spleen cells were cultured with CRA (20 µg/ml) or FRA (12 µg/ml) and Con A (2.5 µg/ml). Supernatants were collected 30 days post-infection with the Y strain of *T. cruzi*. Each bar represents the average values ± standard deviations obtained from duplicate cultures

ity of the red pulps was found in the spleens, with great numbers of polimorphonuclear cells, mainly eosinophils, as well as immature blood cells from black and white lineages. At 14 d.p.i. slight to moderate acute focal inflammatory infiltrates were observed in the myocardium and epicardium of immunized and non-immunized infected mice. These lesions, although similar in both mouse strains, appeared to be less intense in CRA-immunized C57BL/6, as compared to the BALB/c mouse strain. FRA-immunized BALB/c mice had only very slight and scarce inflammatory infiltrates, while CRA-immunized animals showed some focal atrial



inflammation. At 30 d.p.i., less severe cardiac and splenic inflammatory lesions could still be detected in control animals, but only a few foci of interstitial myocarditis and epicarditis were observed in CRA- and FRA-immunized BALB/c mice. In FRA-immunized C57BL/6 mice, cardiac lesions were negligible.

Discussion

The successful survival of protozoan pathogens such as *T. cruzi* depends mainly on evading the host immune system by, for example, increasing phagocytic activity or anergy of T cells (Zambrano-Villa et al. 2002). Besides, parasitic infections frequently result in high $CD4^+$ T cell responses characterized by Th1 or Th2 cytokine production profiles. This polarization may be brought about by different factors of pathogen-antigen-presenting-cell interaction (Jankovic et al. 2001). In order to evaluate which are the polarizing cytokines or what kind of immune response this interaction produces, investigators have studied several *T. cruzi* antigens (Frasch et al. 1991; Franco da Silveira et al. 1992; Taibi et al. 1995; Santori et al. 1996; Pereira-Chioccola et al. 1999). Different murine models have been used for this purpose. Some strains of inbred mice survive acute infection with *T. cruzi*, while others die (Wrightsman et al. 1982; Andrade et al. 1985; Silva et al. 1992).

In the present paper, the induction of immune response in both BALB/c and C57Bl/6 mice immunized with CRA and FRA recombinant antigens of *T. cruzi* challenged with the Y strain of the parasite was investigated. The analysis of the humoral immune response revealed that both strains of challenged mice were able to induce high levels of IgG1, IgG2a, IgG2b and IgG3 in the initial phase of the infection. FRA-immunized C57BL/6 mice produced only the IgG2b isotype. However, high levels of all isotypes were observed at 30 d.p.i. only with CRA-immunized C57BL/6 mice. Since IgG1 and IgG2b are associated with Th2 and IgG2a and IgG3 are associated with Th1 responses, the lack of detectable amounts of parasite-specific antibodies could be due to down-regulation of T and B cell responses in these animals resulting from IL-10 and IFN-γ produced by trypanosome-specific regulatory cells. In the current study, IL-10 and IFN-γ production were observed in both strains of mice. These findings may explain the lack of detectable amounts of antibodies.

It is well established that the acute phase of murine *T. cruzi* infection is initially associated with polyclonal activation. This activation is basically characterized by an intensive B cells proliferation and is not specific to parasite antigens (Minoprio et al. 1988). On the other hand, the differentiation of B cells is very efficiently stimulated when an antigen is presented in a repetitive rigid form, such as that found on the surface of infectious agents (Zinkernagel and Hengartner 2001). CRA and FRA present repetitive structures and were capable of inducing specific antibodies in BALB/c and C57BL/6 mice even in the absence of *T. cruzi* infection (Pereira et al. 2003a, 2003b). In the present paper, although antibodies involved in the elimination of the parasites, as reported by Pereira et al. (2003a, 2003b), were detected; and despite the survival of a significant number of immunized infected mice when compared with the control group, the humoral immune response was not enough to provide full protection against *T. cruzi* at 30 d.p.i.

The acute phase of *T. cruzi* experimental infection is also characterized by severe immune supression of T and B cells to mitogens and to parasite antigens, with partial restoration of the immune response in the early chronic phase (Curotto de Lafaille et al. 1990). These investigators showed that *T. cruzi*-specific proliferative responses of T cells can be detected in lymph nodes throughout the course of the infection. In our report, cellular proliferation was minimal in both strains of mice immunized with CRA and FRA. The impaired immunological response of acutely infected mice may be partially due to the absence or marked reduction of responder and/or accessory T lymphocytes. According to Motrán et al. (1996) the deactivation of accessory T cells during intracellular infection by *T. cruzi* could justify the supression caused by this parasite. Deactivation may occur due to down-regulation of key molecules involved in T-cell activation and proliferation, induction of autoinhibitory cytokines such as IL-10 and the transformation of growth factor β (TGF-β) or impaired secretion of monocyte derived factors involved in co-stimulatory signals (Motrán et al. 1996).

In the present trial, high levels of IFN-γ were produced by both mouse strains when speen cells were stimulated in vitro with CRA or FRA recombinant antigens, although a proliferative response was not detectable. Previously reported studies showed that IFN-γ has been most closely associated with host resistance during the acute phase of infection (Vespa et al. 1994; Silva et al. 1995; Cardillo et al. 1996). Muñoz-Fernández et al. (1992) suggest that IFN-γ and TNF-α, secreted by *T. cruzi*-immune T cells, are involved in the activation of the trypanocidal activity of mouse macrophages through an NO-dependent mechanism. In the present paper, the production of IFN-γ and TNF-α did not attain appropriate levels to control infection against *T. cruzi*. Quantification of bloodstream trypomastigotes showed differences in the parasite burden between the two mouse strains. Parasitemia was lower in CRA- or FRA- immunized C57BL/6 than in BALB/c mice. These results indicate that the interplay between host and parasite genetic differences may influence the outcome of a mouse infection with *T. cruzi*.

The above mentioned differences between C57BL/6 and BALB/c mice were not surprinsing. However, the survival rate (Table 1) was unexpectedly high in CRA- or FRA-immunized BALB/c mice. This finding suggest that CRA and FRA antigens were able to induce an efficient protective response, mainly in BALB/c mice, considered to be a susceptible mouse strain. Survival

rates of 60% and 83.3% were detected for CRA-immunized BALB/c and for CRA-immunized C57BL/6 –mice, respectively. FRA-immunized BALB/c mice had a survival rate of 50%, while no significant survival rate was observed in C57BL/6. It is likely that enhanced resistance, as measured by an increased survival rate, may be associated with synthesis of IFN-γ. It has been well demonstrated that the protective response is more frequently associated with a Th1 cytokine profile (Miller et al. 1996).

It is possible that the immunization schedule used with CRA and FRA antigens did not induce significant levels of protective response against a lethal challenge with the parasite. It has been demonstrated that IL-10 is associated with susceptibility to *T. cruzi* infection (Silva et al. 1992) and the treatment of IL-10 KO and WT mice with recombinant IL-10 results in increased parasitemia (Abrahamsohn et al. 1996). In the current trial, the levels of IL-10 were similar between experimental and control groups. This may have been enough to inhibit the action of IFN-γ. One of the alternatives to induce the immune protection could be the in vivo administration of IFN-γ or IL-12 in association with CRA or FRA antigens. In vivo administration of recombinant IFN-γ induces macrophage activation and prevents acute disease, immune supression, and death in mice experimentally infected with *T. cruzi* (Reed 1988). Furthermore, interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for IFN-*gamma* production (Seder et al. 1993) and acts to control parasite replication during acute infection of mice (Aliberti et al. 1996). Thus, different strategies in the immunization of mice, mainly with CRA, could generate a more efficient immune response.

The present results corroborate the idea of the use of IFN-γ cytokine as a vaccine adjuvant and provide new insights for the development of strategies for beneficial endogenous immune response and disease treatment. Although immunization with CRA or FRA antigens has failed to produce complete immunity, we are currently attempting to design other vaccination protocols to improve protection. The association of CRA and FRA antigens with recombinant IFN-γ could be evaluated in the future.

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