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Evaluation of the immune response to CRA and FRA recombinant antigens of *Trypanosoma cruzi* in C57BL/6 mice

Avaliação da resposta imune em camundongos C57BL/6 imunizados com os antígenos recombinantes CRA e FRA de *Trypanosoma cruzi*

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Abstract *Humoral and cellular immune responses were evaluated in 44 C57BL/6 mice immunized with the* Trypanosoma cruzi *recombinant antigens CRA and FRA. Both antigens induced cutaneous immediate-type hypersensitivity response. The levels of IgG1, IgG2a, IgG2b and IgG3 were high in CRA immunized mice. IgG3 was the predominant isotype. Although no difference in antibody levels was observed in FRA-immunized mice when compared to control mice, both antigens were able to induce lymphoproliferation in immunized mice. Significant differences were observed between incorporation of [^{3}H] – thymidine by spleen cell stimulated* in vitro *with CRA or FRA and the control group. These results suggest that CRA and FRA could be involved in mechanisms of resistance to* Trypanosoma cruzi *infection.*

Key-words: Trypanosoma cruzi*. Recombinant antigens. Immunization. Isotypes. Lymphoproliferation.*

Resumo *As respostas imune humoral e celular foram avaliadas em 44 camundongos C57Bl/6 imunizados com os antígenos recombinantes CRA e FRA de* Trypanosoma cruzi. *Ambos antígenos induziram reação de hipersensibilidade do tipo imediato. Os níveis de IgG1, IgG2a, IgG2b e IgG3 foram elevados nos camundongos imunizados com CRA. IgG3 foi o isotipo predominante. Nenhuma diferença nos níveis de anticorpos foi observada em camundongos imunizados com FRA em relação aos animais controle. No entanto, ambos antígenos foram capazes de induzir proliferação de linfócitos em camundongos imunizados. Diferenças significativas foram observadas entre a incorporação da timidina – [^{3}H] pelas células esplênicas estimuladas com CRA ou FRA e o grupo controle. Esses resultados sugerem que CRA e FRA poderão estar envolvidos nos mecanismos de resistência à infecção pelo* Trypanosoma cruzi*.*

Palavras-chaves: Trypanosoma cruzi. *Antígenos recombinantes. Imunização. Isotipos. Linfoproliferação.*

Chagas' disease, caused by *Trypanosoma cruzi*, is endemic in several countries in Latin America. Despite initiatives to interrupt vector transmission and improvements in serum screening in blood banks, it is estimated that 16-18 million people are infected and at least 90 million are estimated to be at risk of infection (WHO)[22]. Due to the high prevalence of this disease and the absence of an effective treatment option, immunotherapy strategies aimed at the elimination of the parasite by the host have been gaining increased importance.

Previous studies have demonstrated that the immune response plays an important role in protection against infection with *Trypanosoma cruzi* in mice[6 10 15]. Murine infection has been used extensively as an experimental disease model since these animals develop detectable parasitemia during acute infection followed by chronic tissue infection. Patterns of susceptibility and resistance to *T. cruzi* are dependent on the parasite and mouse strains[12 20].

Recombinant DNA technology has enabled the molecular cloning of several genes encoding antigenic

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T. cruzi proteins. Cloned segments of *T. cruzi* genes have been used to produce portions of antigenic proteins in bacteria, and several of these have been used as antigens in serodiagnosis[7 11] or immunoprotection[16 17] assays. Recombinant antigens have been used as tools for analyzing the immune response towards the parasite. These antigens are chemically defined and easy to produce, enabling the assay of various adjuvant and immunizing protocols.

The aim of this study was to evaluate the humoral and cellular immune responses in C57BL/6 mice immunized with two recombinant antigens of *T. cruzi* (CRA and FRA) and apply them to future assays of protection against the parasite. These antigens have been used, successfully in serological diagnosis of Chagas' disease[7 11 18]. CRA (cytoplasmic repetitive antigen) is detected in epimastigote and amastigote forms while FRA (flagellar repetitive antigen) is found in both epimastigote and trypomastigote forms[12].

MATERIAL AND METHODS

Forty-four male C57Bl/6 mice (6-8 weeks old) were obtained from the Fundação Oswaldo Cruz colony (Rio de Janeiro, Brazil). The mice were used in accordance with the Ethical Committee for the Use of Experimental Animals guidelines from the Fundação Oswaldo Cruz/ FIOCRUZ (Ministry of Health, Brazil).

The CRA and FRA recombinant proteins (a kind gift from Antonio Ferreira and Edimilson Silva, Laboratório de Reativos/Bio-Manguinhos/FIOCRUZ) were produced in *Escherichia coli* transformed with plasmid pQE 30 (Qiagen). The purity of these recombinant proteins was determined by SDS-10% polyacrylamide gel electrophoresis. Bands of 50 and 30kDa corresponding to CRA and FRA, respectively, were visualized on the gel silver stained. No band was visualized when the gel was stained with periodic acid-Schiff.

Two groups of 13 mice in each group were immunized with three doses of CRA (20μg) (Group 1 - G1) and FRA (12μg) (Group 2 - G2), respectively (equivalent CRA numbers of FRA molecules), by subcutaneous route in 20-day intervals. The first injection was emulsified in complete Freund's adjuvant and the following immunizations in incomplete Freund's adjuvant. The control group of 18 mice were injected with PBS and adjuvant compounds.

Ten days after the last immunization dose 5 mice from G1 and G2 and their control groups, G1-C and G2-C, respectively, were submitted to cutaneous testing. 25μl PBS containing 5μg of CRA or 3μg of FRA were injected in one hind footpad of G1/G1-C and G2/G2-C respectively and 25μl of PBS in the other footpad as the injection control. Footpad thickness was measured with a caliper (Mitutoyo-Japan) 2, 6, 12, 24, 48 and 72h after challenge with these antigens. The results were reported as the difference between the swelling of the footpad injected with antigen and the swelling of the footpad injected with PBS, and are expressed as the arithmetic mean thickness ± standard deviation (SD).

Fifteen days after the first and the third immunizations serum from 5 individual mice of each group were tested for IgG1, IgG2a, IgG2b, and IgG3 isotypes. After the optimum concentration was determined by checkerboard titration, micro-titer plates (Nunc-Immuno Plates, MaxiSorp, 96 wells, Nalge Nunc International Corporation) were coated with 1μg/ml of CRA or FRA (100μl/well) diluted in 0.05 M Na_2CO_3 buffer, pH 9.6 and incubated overnight at 4°C. The plates were blocked for 2h with PBS-Tween 20 (0.05%) (PBS-Tw) containing 5% fat free milk (Nestle), prior to incubation with 100μl of sera diluted (1:100) in PBS-Tw (overnight, 4°C). The bound antibodies were detected by incubation with peroxidase-conjugated isotype-specific rabbit anti-mouse immunoglobulin (Caltag). The immune complexes were revealed by addition of orthophenyldiamine-OPD and H_2O_2. The reaction was stopped with H_2SO_4 2.5N and the plates were read at 490nm on an automated ELISA reader (Bio-Rad 3550).

Twenty days after the last immunization the spleen cell suspensions of 3 mice from each group were pooled. The cells were cultured in 96-well plates at a density of $4x10^5$ cells/well, in RPMI-1640 containing 10% of fetal calf serum (FCS), 2mM L-glutamine, 1Mm sodium pyruvate and antibiotics (streptomycin=100U/ml and penicillin=100g/ml) (Sigma Chemical Co., St Louis, MO). The cultures were stimulated *in vitro* with CRA (1.25μg/ml and 2.5μg/ml), FRA (1.25μg/ml and 5μg/ml), the mitogen Con A (2.5μg/ml) or maintained in culture medium alone for 72h at 37°C, in an atmosphere of 5% CO_2. The cultures were pulsed with 0.5μCi/well of [^{3}H] – thymidine (Amershan Estou na duvida se deve ser Amersham) for 18h. At the end of the incubation period the lymphocytes were collected with the aid of a semi-automatic cell harvester and the incorporated radioactive thymidine measured by liquid scintillation. The results are expressed as average of triplicate cultures ± SD of the mean.

The Mann-Whitney *U*-test for nonparametric distributions was used to analyze the data. The differences were considered statistically significant when the *P* value was less than 0.05.

RESULTS

The data showed that mice immunized either with CRA or FRA developed cutaneous hypersensitivity reactions (Figure 1). CRA immunized mice showed a significant immediate-type hypersensitivity (ITH) reaction at 2h ($p<0.05$) which remained for 4h following antigenic challenge and which by 12h had faded

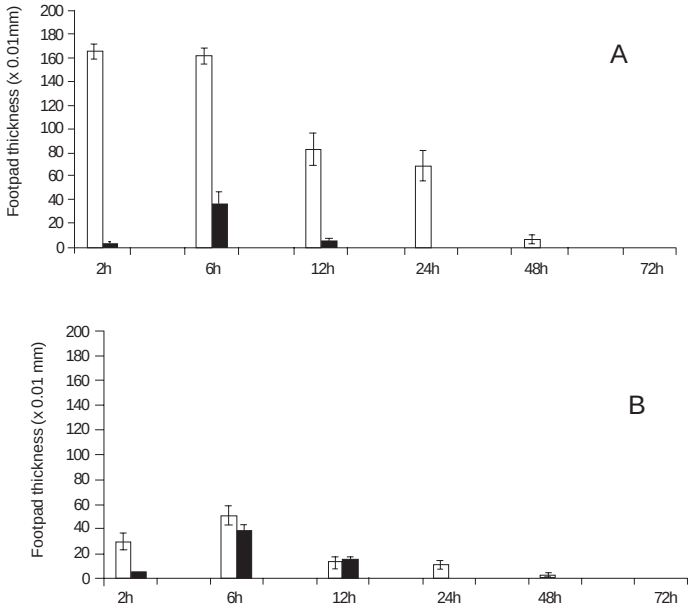

Figure 1 - Cutaneous hypersensitivity reaction in mice immunized with CRA (A) or FRA (B) recombinant antigens of Trypanosoma cruzi. *Open and striped bars represent immunized and control mice, respectively. Each bar represents the mean of the footpad swelling ± SD of five mice.*

drastically when compared to the control mice. FRA immunized mice also showed a significant ITH reaction at 2h ($p<0.05$). Swelling of the footpad was smaller than that observed in CRA immunized mice at the same time points. No significant difference between FRA immunized and control mice was observed after 6h (Figure 1). ITH induced by CRA was eight times greater than that induced by FRA (Figure 1A and B) at 2h after injection of the antigen in the footpad.

The kinetics of *T. cruzi* specific antibody levels for each IgG isotype are shown in Figure 2A, B, C and D. Fifteen days after the 1st (Figure 2A) and 3rd (Figure 2B) immunizations the levels of all IgG isotypes in CRA-immunized mice were significantly greater

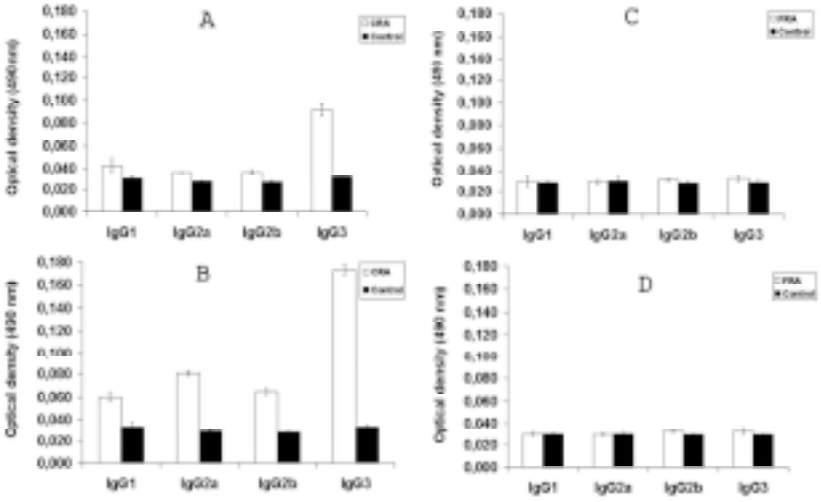

Figure 2 - Kinetics of immunoglobulin G isotypes in mice immunized with CRA or FRA recombinant antigens of Trypanosoma cruzi. *A and B: isotype profile of CRA-immunized mice 15 days after the first and third immunization dose, respectively. C and D: isotype profile of FRA-immunized mice 15 days after the first and third immunization dose, respectively. Open and striped bars represent immunized and control mice, respectively. These results are expressed as the average of five mice ± SD.*

($p<0.05$) than when compared to the values observed in control mice (IgG3 > IgG2a > IgG2b ≥ IgG1). Although all immunoglobulin isotypes increased after the third immunization, it is clear from Figure 2A and B that IgG3 is the major isotype induced by immunization with CRA. This is in contrast to the controls where no difference in antibody levels was observed in FRA immunized mice (Figure 2).

Analysis of the cellular response was evaluated by *in vitro* stimulation of spleen cells with the recombinant antigens. The data obtained is presented in Figure 3A and B. Two different concentrations of the antigens were used, CRA1 and FRA1 (1.25µg/ml) and CRA2 (2.5µg/ml) and FRA2 (5µg/ml). We observed significant difference between incorporation of [^{3}H] - thymidine by spleen cells stimulated with both antigens and controls. Con A induced strong proliferative response (data not shown).

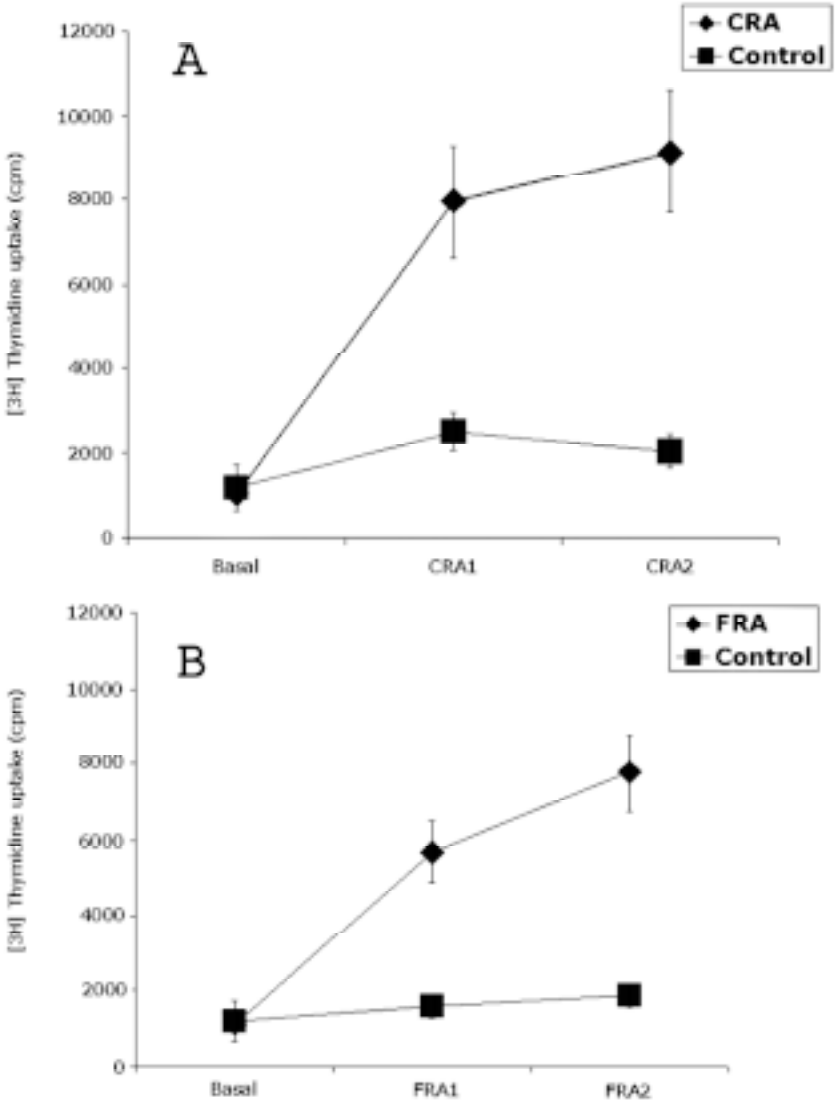

Figure 3 - Lymphoproliferative response of spleen cells from mice (pool=3) immunized with CRA and FRA recombinant antigens of Trypanosoma cruzi. *A: spleen cells stimulated with CRA 1 (1.25µg/ml) and CRA 2 (2.5µg/ml); B: spleen cells stimulated with FRA 1 (1.25µg/ml) and FRA 2 (5µg/ml). Basal represents the cultures maintained in culture alone, without stimulus. The results are expressed as arithmetic mean of c.p.m. ± SD.*

DISCUSSION

In this study the humoral-antibody and cellular proliferative responses of C57BL/6 mice induced by immunization with the recombinant *T. cruzi* antigens CRA and FRA were evaluated. We have previously demonstrated that these antigens can be used for serological diagnosis of *T. cruzi* infection in man[7 13]. However, little is known about their potential role as immunogens or as potential antigens for vaccination. Thus, in this paper we present the data related to the initial evaluation of the immune responses of the resistant C57BL/6 mouse strain. These animals have been previously demonstrated to be resistant to infection by the CL strain of *T. cruzi*.

The analysis of the humoral immune response revealed that the recombinant antigen CRA but not FRA was able to induce significant levels of specific IgG antibody response to the immunizing antigen, where the main isotype was identified to be IgG3. This isotype is efficient activators of complement[19]. It is noteworthy that other isotypes that were evaluated were also present in high levels. Previous studies have demonstrated that partial protection could be obtained by the passive transfer of IgG2a and IgG2b isotypes from infected to naive mice, leading to both a reduced parasitemia and mortality[9 20]. Although we have not performed passive transfer of the different immunoglobulin isotypes induced by CRA, our data suggests its potential as a protective antigen since both IgG2a and IgG2b are induced at high levels when compared to the control. Our data showed that CRA also induced high levels of IgG1. According to Brodskin *et al*[4] IgG1 can have a protective effect through antibody-dependent cellular toxicity, phagocytosis and anaphylactic reactions.

The ITH reaction was very strong in mice immunized with CRA. These results are in agreement with the presence of IgG1 observed in the sera of CRA immunized mice. Immediate hypersensitivity reactions, that develop minutes to a few hours of antigen challenge can be due to anaphylactic antibodies or immune complexes (Arthus reaction)[5]. IgG1 and IgE are the only immunoglobulin isotypes that can elicit active and passive anaphylactic reaction in mice[3 13], through binding to FcεRI and FcγRIII, respectively, on mast cells[7 14]. Further studies are needed to evaluate the role of antibodies in ITH IgE responses. In addition, histopathological analyses must be performed to evaluate the type of cellular infiltrate present in the footpads.

In vitro stimulation of spleen cells from mice immunized with CRA and FRA induced significant cell proliferation to these antigens. The proliferative response was observed to be dependent on the concentrations of CRA and FRA. This demonstrates that the antigens also induce specific cellular immune responses since spleen cells from control mice did not show any significant uptake of [^{3}H] – thymidine in the presence of the same antigenic stimulus. The potential role of the cellular immune response in the induction of protection is being evaluated by determining the cytokine pattern induced by these two antigens. This is important information that, together with the challenge experiments, will indicate the relationship between Type 1 and Type 2 responses and the putative protective immune response induced by CRA or FRA. It will also be interesting to evaluate whether combined immunization with both CRA and FRA offers more potential in inducing a protective immune response than when used singularly. These studies are currently in progress in our laboratory.

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