



Brief Definitive Report

Intravenous immunoglobulin increases survival time in the acute phase of experimental Chagas disease

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SUMMARY

Chagas disease induced by Trypanosoma cruzi (Tc) infection is an important cause of mortality and morbidity affecting the cardiovascular system for which presently available therapies are insufficient and largely inadequate. Intravenous immunoglobulin (IVIg) is a therapeutic preparation containing normal polyspecific IgG obtained from plasma pools of several thousand healthy donors and is used in several autoimmune, inflammatory and infectious diseases. In the study of heart from mice chronically infected with Tc, we observed that IVIg restores type 1 atrioventricular block or bradycardia. In the present study, we investigated the effects of IVIg in acute Tc infection. Intravenous immunoglobulin administration after the first week of infection was associated with an increase in survival time. Taken together, results observed in the chronic and in the acute phase associate IVIg treatment with a favourable outcome in T. cruzi infection.

Keywords acute phase, Chagas disease, intravenous immunoglobulin, mice

INTRODUCTION

Chagas disease (CD), caused by the intracellular kinetoplastid parasite *Trypanosoma cruzi* (Tc), is a widely distrib-

uted debilitating human illness affecting 7.5 million people in Central and South America and is an important cause of mortality and morbidity (1,2). Nonendemic areas such as Europe and US have recently drawn attention to CD for the potential transmission by blood transfusion, organ transplantation or congenital route because of the international migrations. Chagas disease is characterized by an acute phase with patent parasitaemia and a long-lasting chronic phase that remains largely asymptomatic (2). One-third of Tc-infected individuals living in areas where CD is endemic will eventually develop chronic chagasic cardiomyopathy (2). Presently available therapies are inadequate and insufficient (3). Nifurtimox and benznidazole, the only two trypanocide drugs available, have toxic side effects, are not effective for all parasite strains, require long courses of administration and have limited efficacy, especially in the chronic phase (3,4).

Tc infection in mice is a relevant experimental model for understanding the immunopathology of CD and tissue damage (5–9). The early phases of experimental infection are characterized by a number of alterations in the homeostasis of the host immune system such as nonspecific polyclonal B cell activation, hypergammaglobulinemia with elevated autoantibody titres and blockade of T cell activation associated with apoptosis. These typical acute phase features are accompanied by humoral and cellular immunosuppression that contribute to parasite persistence in the host and disease progression (5–9). Parasite persistence is the most important cause of the chronic phase of the infection, but autoimmune events may also contribute to the tissue lesions (2,10,11). The combination of both autoimmune and inflammatory responses in acute Tc infection provides a basis for conception of immunotherapeutic strategies.

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Intravenous immunoglobulin (IVIg) is a therapeutic preparation containing normal polyspecific IgG obtained from plasma pools of several thousand healthy donors (12). In addition to primary and secondary immune deficiencies, IVIg preparations are used in several autoimmune, inflammatory (12–15) and infectious diseases (16,17) including pathological situations implicating cardiac complications (18–21). Physiopathology of chronic chagasic cardiomyopathy involves an autoimmune imbalance that could hypothetically be reverted by IVIg treatment. We tested this hypothesis in chronic mice and observed a reversion of atrioventricular block and/or bradycardia upon infusion of IVIg (BP Olivieri, AP Souza, GM Oliveira, C Britto, SV Kaveri, TC Araujo-Jorge, unpublished results). However, the question regarding the effects of IVIg in the acute phase remained open. The fact that the major parasitological and immunological changes in the Tc infection begin in the acute phase of the disease prompted us to investigate whether IVIg infusion in infected mice could alter the natural outcome in this stage of the infection. Clinical and parasitological parameters were scored to evaluate the effect of IVIg treatment. Our results provide evidence that the progression of infection is indeed altered by two injections of IVIg, 7 days after parasite inoculation, associated with an increase in survival time (ST).

METHODS

Experimental infection and parasitological parameter analysis

Six-week-old female BALB/c mice were inoculated intraperitoneally with 10^3 blood trypomastigotes of *T. cruzi*, CL Brener clone in 200 μ L of saline solution, as described (22,23). All procedures carried out in this work were performed in accordance with the regulations of the animal ethics committee of the Oswaldo Cruz Foundation and to specific guidelines for experimental studies in CD (5). The weight of the animals was followed daily. Different experiments were designed to measure parasitaemia and mortality/ST of each animal (5). The overall mortality rate was monitored until day 30 post-infection. Survival analysis comparing groups of mice was performed according to Gehan's Wilcoxon and Cox's nonparametric tests with the software STATISTICA (StatSoft, Inc., Tulsa, OK, USA).

IVIg treatment and half-life of IVIg in noninfected mice

Sandoglobulin® (CSL-Behring, Bern, Switzerland) was diluted in saline solution and administered intravenously at 1 g/kg. Infected untreated mice were used as controls, receiving saline infusions at comparable time points. Sev-

eral studies with human IVIg have established the value of animal models in the exploration of the mechanisms of action of IVIg (17,20,24–26). On two consecutive days, we administered 20 mg IVIg per mouse weighing 20 g; thus, they received a total amount of 40 mg IVIg. We quantified the levels of human IgG in the serum of uninfected mice that received IVIg infusion to establish its kinetics and half-life. Intravenous immunoglobulin was infused to five young adult BALB/c mice, which were bled before treatment and daily for the first 7 days, and subsequently at days 10 and 20 post-infusion. The serum concentration of human IgG peaked after 24 h, ranging from 20 to 30 mg/mL (data not shown). These levels decreased, reaching 50% of the initial amount 3–4 days post-treatment. They further decreased to about 2 mg/mL on day 7, to 1 mg/mL on day 10 and, by day 20, we were unable to detect human IgG in mouse serum. Considering these data, we estimated that the half-life of IVIg in mouse was 3–4 days. Thus, we chose to infuse IVIg in the infected mice on the 6th and 7th day post-infection, to ensure that the higher IgG levels would coincide with the first parasitaemia peak.

Quantitation of immunoglobulins

An independent experiment was designed to follow the levels of total IgM and IgG in infected and in IVIg-treated mice. ELISA was performed to determine total mouse IgM and IgG as well as human IgG concentrations, according to previous protocols (22,27).

RESULTS

IVIg increases ST in *T. cruzi*-infected mice

To study the effect of IVIg on mortality of Tc-infected mice without any concurrent trypanocide treatment, we chose an experimental model that displayed high mortality rates, with no survivors after 24 days post-infection (BALB/c mice infected with CL-Brener clone of Tc, Figure 1a, group Infected) and performed two independent experiments without bleeding and subjecting animals to any intervention other than measuring the body weight. Contrasting with the Tc-infected and untreated group, mice that had received IVIg infusion presented a significant increase in ST ($P < 0.02$) with a delay in mortality of 4–5 days, suggesting a more resistant phenotype. The cumulative mortality curve (Figure 1a) shows these differences for each consecutive day, depicting that 100% mortality was attained after 25 days for the infected untreated group and after 30 days for the IVIg treated group. This experiment was repeated, and the beneficial effect of IVIg on the delay in mortality was evidenced: ST for these

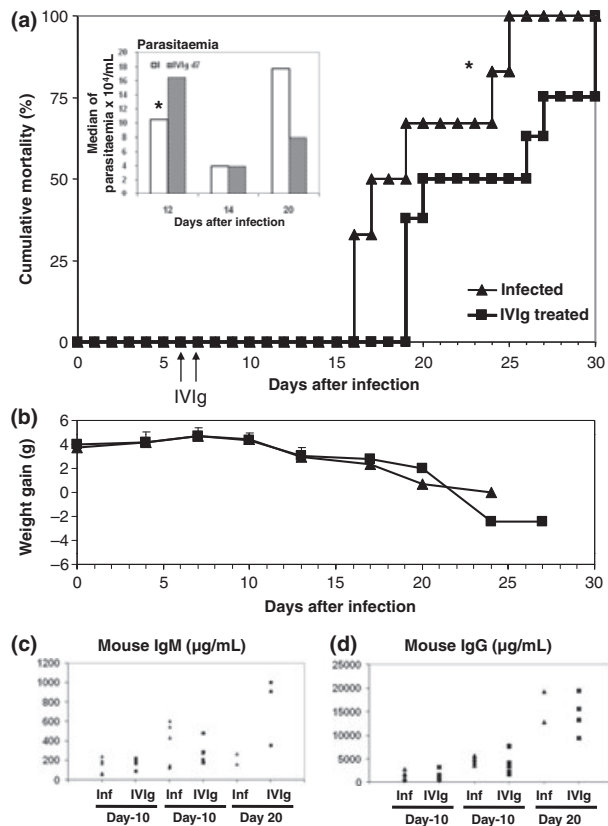


Figure 1 Intravenous immunoglobulin (IVIg) effect on the parasitological, clinical and serological parameters of *Trypanosoma cruzi*-infected mice. (a) Shows cumulative mortality rate and median of parasitaemia (a, inset); (b) Shows mean of body weight gain of mice followed in (a). Intravenous immunoglobulin was infused at days 6 and 7 after infection. (a, b) Show data from a representative experiment of two, starting with six mice in the infected, untreated group and eight mice in the infected IVIg-treated group and ending with no survivors on day 25 and 30, respectively, for each group. (c, d) Show individual serum levels of total mouse IgM (c) and IgG (d), in an experiment starting with six mice/group. Mice were bled at three different time points -10 days before (d-10), on day 10 and 20 days post-infection (d10, d20); two and three mice survived until day 20, respectively, for each group. *T. cruzi*-infected untreated mice are represented as triangles and *T. cruzi*-infected mice infused with IVIg are represented as squares.

experiments were: 19 vs. 23 days, respectively, for infected, untreated ($n = 6$) and infected, IVIg treated mice ($n = 8$) in the first experiment (Figure 1a) and 28.8 vs. 34 days, respectively, for infected, untreated ($n = 8$) and infected, IVIg treated mice ($n = 7$) in the second experiment (not shown).

Concerning parasitaemia, as it is known for the CL-Brener clone characteristics, the first peak was observed on the 12th day post-infection (Figure 1a, inset), and was significantly higher in the IVIg group. It was followed by a decrease on the two consecutive days and further

followed by the characteristic second peak of parasitaemia observed on the 20th day post-infection. A tendency towards a lower parasitaemia in the IVIg group was observed, but the low number of survivors in the untreated group at this point did not allow an appropriate statistical analysis. The evolution of body weight during *T. cruzi* infection was similar in the two groups of mice, with a progressive decrease in weight gain (Figure 1b).

Effect of IVIg treatment on the hypergammaglobulinemia associated with *T. cruzi* infection

Total serum levels of mouse IgM (Figure 1c) and IgG (Figure 1d) were determined in infected animals either treated or not treated with IVIg. As previously described (6–8,22), we observed the typical hypergammaglobulinemia early after Tc infection: both IgM and IgG classes increased progressively after 10 and 20 days. There were no statistical differences between treated and untreated mice at days -10 and 10. Because of the mortality in both groups by day 20, there were only three survivors in IVIg-treated and two in the untreated group thus preventing statistical assessment of the data. However, a marked increase of serum IgM could be observed in the IVIg-treated group after 20 days post-infection in comparison with untreated group (Figure 1c): 163 and 263 µg/mL IgM (mean = 217 + 71) in the two infected mice that survived and 350, 907 and 1000 µg/mL IgM (mean = 752 + 350) in the three survivors in the IVIg-treated group. These results are indicative of an active role of injected IVIg on the immune system. Furthermore, total levels of human Ig in the Tc-infected mice treated with IVIg was about 1 mg/mL by day 10 after infection and undetectable by day 20 (data not shown), revealing that the therapeutic IVIg levels followed a similar decay kinetics to that observed in noninfected mice (data not shown).

DISCUSSION

Intravenous immunoglobulin is currently being used as a therapeutic agent for several autoimmune and inflammatory disorders, and more recently for infectious diseases (16,17,20). In view of the beneficial effect of IVIg that we have observed in chronic chagasic mice (Olivieri *et al.*, unpublished results), restoring type I atrioventricular block or bradycardia, here we demonstrated that IVIg also modulates the outcome of the acute phase of Tc infection, inducing a significant increase in ST. Although the mechanisms that underlie this effect are unknown at present, IVIg treatment may have an impact on the B cell repertoire during infection (12). An in-depth study on natural antibody repertoire in infected mice under IVIg treatment

would contribute for a better comprehension of the implicated mechanisms (28). The expansion or suppression of specific B cell clones could be associated with immunopathology of the acute phase. Indeed, polyclonal activation of B cells that differentiate and mature into antibody-secreting plasmocytes is induced early after infection by parasite polyclonal activators (7,29–32). This phenomenon is stronger in the acute phase of mice models (6–9) and also occurs in humans, as clearly shown by a study of an accidental infection that followed chronologically the increase of different isotypes of Ig (33). The expected humoral specific immune response to Tc antigens evolves in parallel with the hyper-response to B cell mitogens. Besides, humans have protective specific lytic anti-galactose antibodies that are not present in mice (34). Intravenous immunoglobulin could also contain putative lytic and/or neutralizing antibodies among the multiple immunoreactivities.

In the high virulent experimental model that we presently used, any effect of the IVIg therapy should take place at the initial stages of the infection in the absence of specific anti-trypanosome chemotherapy. Accordingly, IVIg therapy beginning after the first week of infection was beneficial with increasing ST. As human IgG concentration decreases within a week, a direct effect such as neutralization of pro-inflammatory cytokines is less likely to explain the beneficial effect observed beyond the half-life of infused IgG. Moreover, death in the acute phase is probably because of tumor necrosis factor- α (TNF- α) shock (35) and/or to a more complex renal–cardiac failure (36). Intravenous immunoglobulin treatment does not seem to interfere with these mechanisms because the difference in weight gain, which reflects indirectly the burst of TNF- α biological activity, is not significant between the treated and untreated groups. Survival in the acute phase is related to nitric oxide levels induced by γ -interferon produced by natural killer (NK) cells and macrophages (37). Given that the second peak of parasitaemia in IVIg-treated mice tends to reflect a lower parasite burden, it is possible that NK-mediated innate responses could be associated with IVIg effect. Direct incubation of bloodstream trypomastigotes with IVIg does not induce agglutination, nor reduction in parasite motility (Olivieri *et al.*, unpublished results), suggesting that the effect of IVIg occurs via interaction with the host immune system. However, it is likely that IVIg contains antibodies against antigens shared among infections agents including *T. cruzi*, and these possibilities are yet to be explored (28).

In the present study, in IVIg-treated mice IgM levels doubled after 10 days and increased by fivefold 20 days post-infection. This IgM increase coincides with the delay in mortality curves and the decrease in parasitaemia. The

further characterization of natural IgM repertoire in this period will help to elucidate whether there is an association among the three parameters.

The development of new therapeutic strategies is a central issue in CD (3,4,38). The description of both autoimmune and inflammatory mechanisms involved in the physiopathology of chronic chagasic cardiomyopathy supports the use of immunomodulatory approaches as therapeutic interventions for CD (39). Recent results with compounds that modulate transforming growth factor β effects (40), chemokine (41) and TNF- α (42), together with our present observations, indicate that the future focus of Chagas cardiomyopathy treatment should rely on efforts to restore the immunological tolerance to self-antigens concurrent with regimens to reduce the parasite load. In this sense, the synergistic effect of a trypanocidal drug and an immunotherapeutic strategy such as IVIg may be proposed as a plausible approach for the treatment of CD, especially in the chronic phase. Further, in view of the possibility that each geographical region is endemic for specific diseases, selection of donors contributing to the pool of IVIg from the entire world rather than from a specific region for preparing IVIg might be advantageous. An alternative approach would be the preparation of a tailor-made IVIg from plasma pools of donors from endemic regions for CD (43,44). The present proof of concept in a murine acute model of the CD warrants further pre-clinical studies in mice to advance to clinical trials in patients.

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CONFLICT OF INTEREST STATEMENT

'None of the authors has any potential financial conflict of interest related to this manuscript.'

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