***Trypanosoma cruzi* induces inflammatory reactions in several tissues. The production of prostaglandin $F_{2\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 , known to regulate the immune response and to participate in inflammatory reactions, was studied in mice experimentally infected with *T. cruzi*. The generation of nitric oxide (NO), which could be regulated by cyclooxygenase metabolites, was also evaluated. In the acute infection the extension of inflammatory infiltrates in skeletal muscle as well as the circulating levels of cyclooxygenase metabolites and NO were higher in resistant C3H mice than in susceptible BALB/c mice. In addition, the spontaneous release of NO by spleen cells increased earlier in the C3H mouse strain. In the chronic infections, the tissue inflammatory reaction was still prominent in both groups of mice, but a moderate increase of thromboxane B_2 concentration and in NO released by spleen cells was observed only in C3H mice. This comparative study shows that these mediators could be mainly related to protective mechanisms in the acute phase, but seem not to be involved in its maintenance in the chronic *T. cruzi* infections.**

Key words: *Trypanosoma cruzi*, Prostaglandin $F_{2\alpha}$, 6-Keto-prostaglandin $F_{1\alpha}$, Thromboxane B_2 , Mice, Nitric oxide

Circulating levels of cyclooxygenase metabolites in experimental *Trypanosoma cruzi* infections

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Introduction

Trypanosoma cruzi is an intracellular parasite that causes Chagas' disease, characterized by a progressive inflammatory reaction mainly affecting the function of myocardial and skeletal muscles as well as the viscera of the digestive tract.¹

The murine experimental infections usually show an acute stage with high parasitemia and prominent inflammatory infiltrates in several tissues, including the heart and skeletal muscles.¹ At present, the mechanisms that induce this inflammatory reaction and its role in the resistance and/or the outcome of the disease are not fully understood. Several pro-inflammatory cytokines that tailor the protective immune response in the acute phase can play a role in its onset. In the early infection, host resistance depends on the T helper type 1 (Th1) protective response,² triggered by interleukin (IL)-12 and IL-18³ that synergistically activate T cells and natural killer cells to produce interferon gamma (IFN- γ).⁴ In turn, IFN- γ and tumor necrosis factor alpha (TNF- α) stimulate phagocytic cells to destroy internalized parasites, mainly through nitric oxide (NO) generation,⁵ resulting in a raise of reactive intermediates from oxygen⁶ and nitrogen.⁷ The production of other inflammatory mediators, such as IL-1⁸ and IL-6,⁹ is also enhanced. IL-12,¹⁰ IFN- γ ,¹¹ TNF¹² and NO⁷ are

involved in host resistance, as demonstrated by their experimental blockade.

In addition, it could be of interest to characterize the increase of other inflammatory mediators, such as eicosanoids, and their contribution to host defense. Prostaglandins (PGs), prostacyclins (PGIs) and thromboxanes (TXs) are products of the cyclooxygenase (COX) pathway that metabolizes the arachidonic acid released from cell membranes. An increased production of TXB₂ and 6-keto-PGF_{1 α} was found in A/J mice acutely infected with *T. cruzi*, Tulahuén strain.¹³ In addition, an increase of seric PGE₂ was detected in C3H mice acutely infected with the CA-1 strain of *T. cruzi*.¹⁴ Eicosanoid production also augmented in the rat acutely infected with *T. cruzi*, Y strain, and was mainly located in lipid bodies of peritoneal macrophages.¹⁵ Alterations in the metabolism of arachidonic acid have also been observed in infections with other intracellular pathogens, such as *Leishmania major*,¹⁶ *Leishmania donovani*,¹⁷ African trypanosomes,¹⁸ *Plasmodium bergeri*,¹⁹ *Histoplasma capsulatum*,²⁰ and *Micobacterium intracellulare*.²¹

The aim of this study was to compare the *in vivo* production of both COX-derived inflammatory mediators and NO in BALB/c and C3H mice infected with *T. cruzi*, Tulahuén strain. In the acute phase, we observed an increased production of PGF_{2 α} ,

6-keto-PGF_{1α} and TXB₂ as well as of NO in both mouse strains. A parallelism was observed between the seric levels of these inflammatory mediators and tissue damage, as evaluated by the areas of inflammatory infiltrates in skeletal muscle. In the acute infection, however, the increase of COX metabolites and NO, as well as the tissue inflammatory reaction, were higher in resistant C3H than in susceptible BALB/c mice, suggesting that the inflammatory mediators studied could be involved in or could enhance protective mechanism(s). In addition, even when the extension of the inflammatory reaction was still important in chronically infected mice, the levels of most of the inflammatory mediators studied were similar to those of non-infected controls; thus, it seems that they were not the major causes of long-term maintenance of tissue inflammation.

Materials and methods

Mice and parasites

Eight-week-old male mice of the BALB/c and C3H/He strains, from our animal facilities, were infected intraperitoneally with 150 bloodstream forms of *T. cruzi*, Tulahuén strain. Parasitemias were determined in 5 µl of tail vein blood and mortality recorded. During the chronic phase, mice were studied at 6–8 months post-infection (p.i.). Strain-matched and age-matched non-infected mice were used as controls.

Sera and spleen cells

Mice were anesthetized with ether, bled and sacrificed by cervical dislocation. Sera were fractionated and kept at –70°C. Spleen cell suspensions, obtained using a Teflon grinder, were washed twice in RPMI medium (Sigma Chemical Co., St Louis, MO, USA) containing 5% fetal calf serum (Gibco BRL, New York, USA), 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin and 100 µg/ml of streptomycin. Red cells were lysed by osmotic shock. Viability of the cell suspensions assayed by trypan blue exclusion was ≥98%. To determine the NO released by these cells they were cultured at $10^6/200$ µl of RPMI–5% fetal calf serum for 48 h in a CO₂ incubator, and the cell free supernatants fractionated and stored at –70°C.

Nitrite plus nitrate (NO₂[–] + NO₃[–])

The levels of total NO were evaluated based on the enzymatic conversion of nitrate (NO₃[–]) to nitrite (NO₂[–]) by nitrate reductase, and nitrites were determined with Griess reagent using a commercial Kit (R&D Systems, Minneapolis, MN, USA). Briefly, appropriate dilutions of sera or spleen cell super-

natants were incubated with nitrate reductase and NADH in a 100 µl final volume, for 30 min at 37°C. Then, 100 µl of Griess reagent was added and, after color development at room temperature, the optical density at 540 nm was measured in an enzyme-linked immunosorbent assay (ELISA) reader (RiaStar Packard, Meriden, CT, USA). Sodium nitrate diluted in culture medium or serum was used as standard for the determinations in supernatants or sera, respectively. The assay sensitivity was <1 µM.

Histology

The quadriceps, the muscle with the greatest density of parasites in the acute infection,²² was examined. As opposed to the odd distribution of parasites in the heart, they were homogeneously distributed in the skeletal muscles. The tissue was fixed in 4% formaldehyde, embedded in paraffin and the slides stained with hematoxylin–eosin. Tissue sections were randomly selected and histocytometry was performed with the aid of an ocular grid. Each section was two-blind observed at 160 ×, and the percentage of area with inflammatory infiltrates recorded in an area of 5 mm².

PGF_{2α}, 6-keto-PGF_{1α} and TXB₂

Arachidonic acid metabolites were determined by specific competitive enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocols. Briefly, samples and standards were incubated with the conjugated eicosanoid–acetylcholinesterase as a tracer, and a specific rabbit antiserum in 96-well plates precoated with mouse antibodies anti-rabbit IgG, overnight at 4°C. Then, plates were washed and Elman's reagent, as the enzyme substrate, was added. Optical density was determined at 405 nm in an ELISA Reader (RiaStar Packard). The assay sensitivities were 13, 8 and 24 pg/ml for TXB₂, PGF_{2α} and 6-keto-PGF_{1α}, respectively.

Statistical analysis

In every case, differences between groups were examined by two-tailed Student *t*-test, using Excel 5.0 software. Discontinuous variables were previously normalized by logarithmic transformation. *p* < 0.05 was considered significant.

Results

In the acute stage of *T. cruzi* infection, starting by the end of the second week p.i., BALB/c and C3H mice showed a prominent area of inflammatory infiltrates

in the skeletal muscle. In the third and fourth weeks p.i., this area was larger in C3H mice than in BALB/c mice (Fig. 1). However, BALB/c were more susceptible than C3H mice, as shown by the four times higher parasitemia. Moreover, in this experimental model, one-half of BALB/c mice had died 23 days p.i., while all C3H mice survived the acute phase.

Circulating COX metabolites PGE_2 , PGI_2 and TXA_2 were evaluated based on the concentrations of their stable products $\text{PGF}_{2\alpha}$, 6-keto- $\text{PGF}_{1\alpha}$ and TXB_2 , respectively.²³ In the third and fourth weeks p.i., during the raise of parasitemia and tissue inflammatory reaction, the concentrations of these mediators increased about two to five times as compared with non-infected mice. The circulating levels were higher in C3H mice than in BALB/c infected mice (Fig. 2). Preliminary results did not show significant changes in the first week p.i.

We also evaluated the NO levels in infected mice, since a remarkable effect of PGE_2 on the immune response is the upregulation of NO production.^{24,25} In both mouse strains, seric NO was found increased during the late acute infection, starting earlier and reaching higher concentrations in resistant C3H mice than in susceptible BALB/c mice (Fig. 3). Spleen cells could be an important source of NO production. We studied the spontaneous release of NO by spleen cells and found different kinetics in both experimental models. In C3H mice the values peaked at 7 days

FIG. 1. Parasitemia and area of tissue inflammatory infiltration (%) in skeletal muscle of acutely and chronically (>180 days p.i.) infected C3H and BALB/c mice. In both mouse strains, the inflammatory infiltrates were higher in infected than in non-infected mice, starting 11 days p.i. ($p < 0.05$, t -test). Means $\pm$ standard errors of 4–6 mice/group are shown. *Differences between mouse strains are significant ($p < 0.05$, t -test).

FIG. 2. Circulating levels of COX metabolites in C3H and BALB/c mice acutely infected with *T. cruzi*. Levels of $\text{PGF}_{2\alpha}$, 6-keto- $\text{PGF}_{1\alpha}$ and TXB_2 were determined in sera collected at the days p.i. indicated. The concentrations were higher than controls on the third and fourth week p.i. ($p < 0.05$, t -test). Means $\pm$ standard errors of 4–6 mice/group are shown. *Differences between mouse strains are significant ($p < 0.05$, t -test).

p.i. and decreased thereafter, while in BALB/c mice the NO reached its highest values in the fourth week p.i. (Fig. 3).

In the chronic stage, the inflammatory infiltrates were still prominent in both mouse strains (Fig. 1). Circulating parasites were no longer detected, although they were occasionally observed in the skeletal muscle. The NO production by spleen cells was still increased in chronically infected BALB/c mice, with similar levels to those observed in the acute infection, whereas normal values were found in C3H mice. Despite this difference, the circulating levels were similar to non-infected controls in both mouse strains (Fig. 3). On the other hand, the levels of circulating COX metabolites in chronically infected mice were similar to non-infected control in both groups, and only TXB_2 was still increased in C3H mice (Fig. 4).

FIG. 3. Levels of $\text{NO}_2^- + \text{NO}_3^-$ in the sera and spleen cells supernatants of acutely and chronically (>180 days p.i.) infected C3H and BALB/c mice. Means $\pm$ standard errors of 4–6 mice/group are shown. In the cell supernatants, the values increased over the non-infected control at 7 days p.i. in C3H mice and at 21 days p.i. in BALB/c mice ($p < 0.05$, t -test). *Differences between mouse strains are significant ($p < 0.05$, t -test).

Discussion

Experimental infection with *T. cruzi*, Tulahuén strain, triggered an intense inflammatory response in skeletal muscle that was larger in C3H mice than in BALB/c mice, despite the higher parasitemia and mortality of the latter mice. In addition, the production of COX-derived inflammatory mediators, which paralleled the parasitemia in the acute phase, was also higher in resistant C3H mice than in susceptible BALB/c mice. These observations suggest that these mediators could play a role in both inflammatory reaction and host protection.

Disparate results have been reported about the relationship of the increased production of COX metabolites and resistance to intracellular infections. In mice infected with *M. intracellulare*, the *in vivo* administration of PGE_2 inhibitors decreased the parasite burden.²¹ Moreover, the *in vivo* production of PGE_2 seems to be detrimental to the control of *Leishmania* infection.^{16,24} *In vitro*, however, PGE_2 enhances the expression of IFN- γ inducible NO synthase (iNOS) and markedly increases the killing of *Leishmania enrietti* or *L. major* by activated macrophages.²⁵

COX metabolites could contribute to both protection and pathology in experimental *T. cruzi* infections. In C3H mice infected with the CA-1 strain the treatment with COX inhibitors enhanced the mortality, and the administration of a PGE_2 analog reversed

FIG. 4. Circulating levels of $\text{PGF}_{2\alpha}$, 6-keto- $\text{PGF}_{1\alpha}$ and TXB_2 in mice chronically infected with *T. cruzi* (>180 days p.i.) (hatched bars) and in age-matched non-infected controls (open bars). Means $\pm$ standard errors of 4–6 mice/group are shown. *Differences with non-infected mice are significant ($p < 0.05$, t -test).

this effect.¹⁴ However, in BALB/c mice infected with the PF strain of *T. cruzi* this increased the platelet reactivity,²⁶ a physiological consequence of the raise of COX metabolites that has been related to both the alterations in the microcirculation of the heart²⁷ and host protection *in vitro*.²⁸

These apparently opposite effects may be related to the role the inflammatory reaction plays in the mechanisms of protection and tissue damage, and hence its relationship to the outcome of the disease. In this regard, the effect of COX metabolites on the Th1 to Th2 balance could be of great relevance. *In vitro*, PGE_2 has no effect on or enhances the production of Th2 cytokines but drastically inhibits the production of Th1 cytokines, biasing the immune system towards a Th2 response.²⁹ Conversely, Th2 cytokines are able to inhibit COX-2 activity,³⁰ and consequently PGE_2 production.³¹ However, it is not clear how the increase of PG can favor the resistance to *T. cruzi* infection that depends mainly on the Th1 response.² In this regard, the time course of this response could offer a possible explanation. Only the very early onset of the Th1 response, with IFN- γ secretion, is important for the host protection^{11,32} and PGs increased in the late acute phase when the production of pro-inflammatory Th1 cytokines has to be controlled to protect the host from the induction of tissue damage.

The production of COX metabolites and the PGE_2 -induced downregulation of the Th1 response could also explain our previous observations in these

experimental models. The differences in the susceptibility to the infection between BALB/c and C3H mice can be mostly attributed to the early production of IFN- γ in the C3H strain.³³ IFN- γ production increases later on in BALB/c mice, and at the same time they release lower amounts of PGE₂ as compared with C3H mice. Also in the late acute phase, IL-10 production that decreases COX activity³⁰ increases in BALB/c mice.³² An early onset of the Th1 response is important, but also its inhibition in the late acute phase is of great relevance. At this time, PGE₂ release could be an alternative to IL-10 in the downregulation of the response, and the resistant mice produced mainly PGE₂ while IL-10 release predominated in the susceptible BALB/c strain.

Early components of the immune response, such as TNF- α and IL-1, could be a link between the levels of inflammatory mediators and the Th1/Th2 balance with important consequences for host survival. IL-1 is able to increase the production of PG and NO.³⁴ Moreover, in the acute *T. cruzi* infection, TNF- α stimulates PG and NO synthesis, but in the late acute phase they seem to control their own production by inhibiting TNF- α release.^{35,36} The PGE₂ upregulation of NO production^{24,25} could explain the simultaneous increase of circulating arachidonic acid metabolites and NO we found in the late acute phase.

In the acute phase, NO seems to be also involved in the pathology of *T. cruzi* infection. Normal mice infected with *T. cruzi*, Tulahuén strain, have more severe myocardial alterations, greater heart weight and myocardial inflammation than their iNOS-deficient counterpart, indicating that NO affects the development and progression of myocardial dysfunction.³⁷ On the other hand, the early stimulation of NO production by IL-12-induced IFN- γ has a pivotal role in the host defense to *T. cruzi*.³⁸ The production of NO by spleen cells paralleled the previously observed local synthesis of IFN- γ .³³ The increase of IFN- γ release by spleen cell in the first week of the infection in C3H mice³³ could contribute to the earlier and higher production of NO in this strain as compared with BALB/c mice. Similar results have been observed in mice infected with *T. cruzi* Y strain.⁷

Host survival and development of the adoptive immune response seems to depend on the balance between the control of the parasite burden and the deleterious effect of the inflammatory response. In the early *T. cruzi* infection, NO and arachidonic acid metabolites could be related to resistance, but later on to tissue damage. The differences observed in the inflammatory mediators in resistant and susceptible mouse strains reinforce their relevance as protective factors and open new perspectives for understanding Chagas' disease.

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