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The sympathetic nervous system affects the susceptibility and course of *Trypanosoma cruzi* infection

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ABSTRACT

Trypanosoma cruzi (*T. cruzi*) is an intracellular parasite that causes Chagas' disease, a major health problem in Latin America. Using a murine model of infection with this parasite, we have previously shown that corticosterone blood levels are markedly elevated during the course of the disease in C57Bl/6 male mice and that this increase is protective for the host by restricting the production of pro-inflammatory cytokines. Since the hypothalamus-pituitary-adrenal (HPA) axis usually operates in a concerted way with the sympathetic nervous system (SNS), we have now studied whether noradrenergic nerves can affect the course of *T. cruzi* infection and the sexual dimorphism observed in the disease. We found a decreased splenic noradrenaline concentration and content, paralleled by a reduction in noradrenergic nerve fibers in the spleen of infected mice, and increased HPA axis activity. These alterations were more marked in males than in females. When the spontaneous loss of noradrenergic nerve fibers was advanced by chemical sympathectomy prior to infection, males died earlier and mortality significantly increased in females. Chemical denervation did not significantly affect the concentration of specific IgM and IgG_{2a} antibodies to *T. cruzi*, and did not worsen myocarditis, but resulted in increased parasitemia and IL-6 and IFN-γ blood levels. The results obtained in this model of parasitic disease provide further indications of the relevance of interactions between the immune system and the SNS for host defense.

1. Introduction

Trypanosoma cruzi (*T. cruzi*) is an intracellular parasite that causes Chagas' disease, also called American trypanosomiasis, a major health problem in Latinamerican countries, where it currently affects around 10 million people. However, the disease is rapidly extending to Europe, USA, Australia and Japan (Basile et al., 2011; Bonney, 2014; Dutra et al., 2014), becoming a global health problem in this century. In humans, the disease occurs in two phases: an acute stage, manifested shortly after the infection, and a chronic one that may develop over 10 years. Chronic infections result in neurological disorders, damage to the heart muscle, and sometimes dilation of the digestive tract (megacolon and megaesophagus). Left untreated, Chagas' disease can be fatal, in most cases due to the cardiac sequelae. We have developed a murine model of *T. cruzi* infection based on the inoculation of only 200 parasites into C57Bl/6 mice, which leads to a progressive and lethal disease in males, with profound thymic atrophy and loss of $CD4^+CD8^+$ thymocytes, and increased levels of pro-inflammatory cytokines and antibodies against parasite antigens (Roggero et al., 2002; Perez et al., 2005). A strong stimulation of the hypothalamus-pituitary-adrenal (HPA) axis was also detected in infected C57Bl/6 mice, as indicated by a more than 10-fold increase in corticosterone blood levels (Roggero et al., 2006). Interference of glucocorticoid effects, either by administration of a glucocorticoid receptor antagonist or following adrenalectomy, results in increased levels of pro-inflammatory cytokines and in earlier death of infected male mice. These results indicate that neuro-endocrine mechanisms are relevant for the susceptibility and course of the disease caused by *T. cruzi*.

The HPA axis usually operates in a concerted way with the sympathetic nervous system (SNS), and noradrenaline (NA), the main neurotransmitter released by sympathetic nerves, can affect different types of immune cells and immune responses in a selective way (for review del Rey and Besedovsky, 2008). On these bases, we have now studied the possibility that the SNS influences the immune response to *T. cruzi* and the course of the disease induced by this parasite in mice. For this purpose, we have first evaluated NA concentration in the spleen of mice infected with *T. cruzi* and

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the consequences that interfering with the activity of the SNS have for the course of the disease. Our initial studies were done in males. When we extended our studies to females, a significant proportion of mice survived after inoculation of the same number of parasites that is lethal for males. Thus, we also analyzed whether there were differences in the SNS of *T. cruzi*-infected male and female mice, and studied the possibility that sympathectomy affects the course of the disease and the sexual dimorphism in the susceptibility to infection with the parasite.

2. Materials and methods

2.1. Parasite, mice, and infection

The Tulahuén strain of *T. cruzi* used in this study was maintained by serial passages in Balb/c suckling mice. C57Bl/6 and Balb/c mice were bred at the animal facilities of the Medical Faculty of Rosario, Argentina. Mice were housed individually for one week before experiments were started and kept single-caged throughout the experiments in temperature-, humidity- and light (12 h cycles)-controlled rooms. Two hundred trypomastigotes suspended in 100 µl of physiological saline (0.9% NaCl) were injected subcutaneously (s.c., 50 µl in each flank) when mice were 8–10 week-old. Control mice received the same amount of physiological saline. The studies were approved by the responsible agency (Ethical Committee for Scientific and Technical Research, CEICyT-UAI, Argentina).

2.2. Evaluation of parasitemia

Bloodstream forms of *T. cruzi* were counted under standardized conditions by direct microscopic observation of 5 µl heparinized blood obtained from the tip of the tail. Data are expressed as number of parasites/50 fields in 40× magnification.

2.3. Sympathetic denervation

Groups of mice were sympathetically denervated at birth by intraperitoneal (i.p.) injection of 6-hydroxydopamine hydrochloride (6-OH-DA; Sigma-Aldrich; 150 mg/kg dissolved in 0.01% ascorbic acid; 1 injection per day over 5 consecutive days, starting when mice were less than 24 h old). Controls received the vehicle alone.

2.4. Corticosterone determination

Plasma samples for hormone determinations were obtained from the tip of the tail under light ether narcosis between 8 and 10 a.m. before (day 0) and 10, 14 and 17 days post infection (p.i.). Blood samples were also obtained from age- and sex-matched controls subjected to the same experimental conditions. Plasma corticosterone levels were determined by ELISA, using the kits and prescriptions provided by the manufacture (IBL International GmbH, Germany).

2.5. NA and adrenaline (A) determinations

NA, precursor and metabolite determinations in the spleen, and catecholamine concentrations in the adrenal gland and plasma were performed by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (del Rey et al., 2006), using the supernatant of tissue samples homogenized in 0.4 M $HClO_4$. Blood plasma samples underwent a purification step using alumina adsorption prior to HPLC determination. Quantification was done by peak height evaluation using an evaluation software (Chromeleon version 6.08; Dionex, Sunnyvale, CA).

2.6. Spleen cellularity

The spleen was weighted and cell suspensions were prepared from approximately one-third of the organ and counted in a Neubauer chamber at appropriate dilutions.

2.7. Tyrosine-hydroxylase staining by immunofluorescence

Spleens were removed 17 days after infection, embedded in Tissue-Tek (Miles Inc., Elkhart, USA) and frozen in liquid nitrogen. Three serial 10 µm-sections from the proximal third of the spleen of 5 mice per group were cut using a freezing microtome, fixed in acetone, and washed three times with PBS. Tissue sections were incubated with sheep anti-tyrosine hydroxylase (Chemicon, USA) overnight at 18 °C, followed by 2 h incubation at 4 °C. After washing with PBS, sections were incubated with donkey anti-sheep-biotin and streptavidin FITC (Dianova, Hamburg, Germany) for 45 min at 25 °C. No binding was detected in the absence of the primary antibody. Following PBS washes, the sections were mounted in 25% glycerol/75% PBS. Ten fields per section were examined with a confocal microscope (Nikon eclipse TE2000-E inverted microscope, D-eclipse C1si, Melville, New York), and representative fields were photographed.

2.8. Evaluation of myocarditis

Evaluation of myocarditis was performed as previously described (Roggero et al., 2002) with slight modifications. Briefly, hearts were removed on day 17 p.i., divided transversally into two parts, and fixed in Bouin-Hollande. Paraffin-embedded 5 µm sections were stained with haematoxylin and eosin. Foci of myocarditis were evaluated by an experienced pathologist blinded to the study groups, and scored as follows: a) small-sized foci, slight infiltration with damage of one or two myocardial fibers (score 1); b) medium-sized foci, aggregated infiltrates compromising three to five muscle fibers (score 2); and c) large-sized foci, heavy accumulation of lymphocytes and macrophages with destruction of more than five muscle fibers (score 3). Results are expressed as total myocarditis score ± SE, calculated by multiplying the number of lesions by the corresponding individual score, and evaluated in whole two slices (each slice obtained from one half of the heart).

2.9. Determination of cytokine levels in serum

Mice were bled by cardiac puncture 17 days p.i. Blood was collected in sterile, endotoxin-free tubes and kept refrigerated until centrifugation. Serum was stored frozen at −20 °C until used. TNFα, IL-1β, IL-6, and IFN-γ concentrations were evaluated by ELISA, using commercially available kits (BD Pharmingen, San Diego, USA). All samples were assayed in duplicate. The limit of quantification (LOQ) of each cytokine was 15.6 pg/ml, except for IL-1β, for which the LOQ was 31.3 pg/ml.

2.10. Determination of T. cruzi-specific IgM and IgG_{2a} antibodies

Specific anti-*T. cruzi* IgM and IgG_{2a} concentrations were measured by ELISA. In brief, microtiter plates (Lockwell Modules, Nalge Nunc International, Naperville, IL, USA) were coated with 20 µg/ml of an epimastigote lysate from the Tulahuén strain, in 0.05 M carbonate–bicarbonate buffer (pH 9.6), blocked with skimmed milk, and incubated for 4 h at 37 °C with a 1:100 dilution of sera from infected or control mice. Specific IgM and IgG_{2a} isotypes were