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Immunoneuroendocrine alterations in patients with progressive forms of chronic Chagas disease

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ABSTRACT

We studied the features of parallel immunoneuroendocrine responses in patients with different degrees of chronic Chagas myocarditis (indeterminate, mild/moderate or severe). A systemic inflammatory scenario was evident in patients with severe myocarditis compared to healthy subjects. This was paralleled by a disrupted activation of the hypothalamus-pituitary-adrenal axis, characterized by decreased concentrations of dehydroepiandrosterone-sulfate (DHEA-s) and an unbalanced cortisol/DHEA-s ratio, reinforcing the view that severe Chagas disease is devoid of an adequate anti-inflammatory milieu, likely involved in pathology. Our study constitutes the first demonstration of neuroendocrine disturbances, in parallel to a systemic inflammatory profile, during progressive human Chagas disease.

1. Introduction

Chagas disease is a major cause of morbidity and mortality in Latin American countries, with nearly 7 million people being affected (WHO, 2007). The acute phase of Chagas disease begins after the incubation period and symptoms can persist approximately two months if untreated. Patients then evolve to a chronic infection, although most of them remain asymptomatic, in the so called indeterminate form of chronic infection. Despite the presence of specific immunity, indeterminate individuals remain infected since some parasites may evade the immune response, and focal inflammatory lesions being found in several tissues. Twenty to thirty percent of the chronically-infected individuals progress to overt disease, with the development of different degrees of cardiac disturbances (Andrade, 1999). Nevertheless, the pathogenetic mechanisms related to the generation of different disease manifestations — including chronic myocarditis — are highly heterogeneous and are not fully understood, motivating many studies to analyze the factors involved in the morbidity of disease (Cunha-Neto et al., 2006; Tarleton, 2001). Investigating the correlation between potential immunoneuroendocrine abnormalities and the diverse clinical manifestations in Chagas disease may provide additional clues in this regard.

The strong interconnection between immune and neuroendocrine systems may optimize the defensive response of the host, but also set the basis for an altered regulation of inflammation; for example, when pathogens cannot be cleared. In such cases, both systems may influence each other, leading to the generation of an unsuitable *milieu* for both infection resistance and host status (Besedovsky and del Rey, 1996; Pérez et al., 2009). In this context, the endocrine activity that parallels immunologic changes, as well as the possible relevance that this integrative immunoendocrine response may have in the course of Chagas disease remains poorly characterized at the experimental level and has not been previously studied in humans. Additionally, our previous studies support the occurrence of disturbed immunoendocrine interconnections in experimental acute *Trypanosoma cruzi* infection. In fact, although hypothalamic-pituitary-adrenal (HPA) axis activation acts as a protective and counter-regulatory mechanism, a cytokine unbalanced milieu driven by HPA axis activation seems to strongly influence the infection outcome (Corrêa-de-Santana et al., 2006; Roggero et al., 2006). As such, disturbances in immunoneuroendocrine circuits might contribute to the pathophysiology of the human disease. Herein, we explored immune and endocrine status in patients with chronic *T. cruzi* infection, and found that certain patterns

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of immunoneuroendocrine environment are associated with the degree of myocardial involvement.

2. Materials and methods

2.1. Patients

The study population consisted of 43 *T. cruzi* chronically infected patients of both sexes and 18 sex and age-matched healthy volunteers (Co), recruited at the Chagas Disease Service from the Department of Cardiology, Hospital Provincial del Centenario de Rosario, National University of Rosario (Rosario, Argentina). None of these patients was under specific treatment (i.e. Benznidazole or Nifurtimox) nor had concomitant pathological disorders. Exclusion criteria comprised neuroendocrine disturbances, immunological diseases, treatment with hormones or immunomodulators. Control subjects were seronegative to *T. cruzi*-specific test. Participants gave their informed consent and the protocol was approved by the Ethics Committee of the National University of Rosario Medical School. The diagnosis was based on at least two positive serological findings (either by ELISA, hemagglutination or immunofluorescence), together with clinical symptoms, heart/chest X-ray, and 12-lead resting electrocardiogram (ECG). Routine laboratory studies were also included.

Chronic chagasic patients with different degrees of cardiac involvement were grouped into three categories: *Indeterminate group* (IND, $n=17$), symptomless, normal ECG and chest X-ray; *Mild to Moderate cardiac group* (M, $n=13$), no congestive heart failure with ECG showing any of the following alterations: incomplete right bundle branch block or complete right bundle branch block, ventricular arrhythmia or chest X-ray cardiothoracic ratio <0.55; and the *Severe cardiac group* (SEV, $n=13$), bearing congestive heart failure, pathological ECG profiles and chest X-ray cardiothoracic ratio >0.55. The mean ± standard error of age for control individuals was 49.9 ± 2.9, for IND 46.0 ± 3, for M patients was 52.9 ± 2.4 and for SEV patients was 54.0 ± 3.7.

2.2. Plasma collection and cytokine, hormone and creatine kinase assays

Blood samples from patients and healthy volunteers were collected between 8:00 and 10:00 a.m. Plasma was obtained from EDTA-treated blood. Samples were centrifuged at 2000 rpm during 30 min and then preserved at −70 °C.

Plasma cytokine concentrations were determined using commercially available ELISA Becton Dickinson/Pharmingen kits (CA, USA), except to IL-6 and IL-17 by R&D (MN, USA). The detection limits for these assays were the following: IFN-γ: 4.7 pg/ml; IL-6: 0.1 pg/ml; IL-17: 15 pg/ml; IL-10: 7.8 pg/ml; TNF-α: 7.8 pg/ml; CCL-2: 7.8 pg/ml; IL-4: 2 pg/ml; and TGF-β: 7.8 pg/ml. All samples were processed individually and assayed in duplicate, with plates being read at 450 nm. Plasma hormones were determined in duplicate by electrochemiluminescence immunoassay (Roche Diagnostics, Basel, Switzerland). Limit of detections were: Cortisol: 2.5 ng/ml; DHEA-s: 0.1 ng/ml; PRL: 2 ng/ml; GH: 0.11 IU/ml; testosterone: 0.07 ng/ml; estradiol: 4.6 pg/ml; T3: 0.2 ng/ml; T4: 0.4 g/dl; IGF-1: 0.15 ng/ml; and Insulin: 0.2 μU/ml. Total creatine kinase (CK) and myocardial CK (CK-MB) were determined by colorimetric test (CK-NAC) and CK-MB test respectively (Wiener Lab, Rosario, Argentina).

2.3. Nitric oxide determination

Nitric oxide (NO) in plasma was measured as nitrite plus nitrate. Briefly, total nitrite was assessed by reducing nitrate to nitrite with a *Pseudomona oleovorans* (strain ATCC 8062) nitrate reductase. Equal amounts of culture supernatants and Griess reagent were combined, and incubated for 10 min at room temperature. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 30% acetic acid and 0.1% naphthylethylene diamine dihydrochloride in 60% acetic acid. Absorbance was measured at 540 m. Nitrite concentration was quantified using various $NaNO_2$ concentrations as standards; data being expressed in micromolar (μM).

2.4. Metabolic variables

The HOMA (Homeostasis Model Assessment) index was calculated using fasting plasma concentrations of [glucose (mmol/l) × insulin (μU/ml)]/22.5; determined by glucose-oxidase assay (Glicemia enzimática, AA Wiener Lab, Rosario, Argentina) and electrochemiluminescence method (Roche Diagnostics, Basel, Switzerland), respectively. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2).

2.5. Statistical analysis

Parametric and non parametric tests were used, depending on the characteristics of variables (normal distribution or not, etc.). Comparisons among groups were made in relation to the heart involvement. Categorical variables were analyzed by the χ^2 or Fisher's exact test when applicable, whereas parametric analysis of variance and Student's *t*-test or non parametric Kruskall–Wallis and *U* the Mann–Whitney test were used to evaluate differences in mean values. When appropriate, background factors were controlled by the unconditional logistic regression method. To assess putative effects of other variables on the means, the general linear model was applied. The level of significance was set at $p<0.05$.

3. Results

3.1. Enhanced immune activity in patients with severe heart involvement

First, we determined the serum levels of major systemic mediators produced during this disease, since many of them could affect endocrine mechanisms that, in turn, might influence the chronic myocardial inflammation. We found that TNF-α, IL-17, IL-6, CCL-2, IFN-γ and NO serum levels were higher in SEV patients than the values found in healthy subjects (Fig. 1). Interleukin-10 remained similar in all groups, whereas IL-4 and TGF-β showed an increase between M and SEV state. Interestingly, IL-17 was noticeable in one third of SEV (5/13) patients and in two M (2/13), remaining undetectable in Co and IND individuals. Total CK (data not shown) and CK-MB were within normal range in all groups.

Furthermore, TNF-α/IL-10 and IL-6/IL-10 ratios were significantly increased in SEV patients compared with healthy controls ($p<0.05$, both cases), whereas no substantial differences were observed between Th1/Th2 cytokine ratios (Table 1). Levels of IL-6 correlated positively with TNF-α values (overall; $r_s=0.24$; $p<0.05$), but not with IL-17.

3.2. DHEA-s diminution and enhanced cortisol/DHEA-s are associated with the severity of heart involvement

Previous data from experimental acute infection showed the existence of a protective response activity mediated by the HPA axis. In a clinical chronic scenario, we evaluated the plasma levels of ACTH, cortisol and DHEA-s. The marked inflammatory profile observed in chagasic patients seems to be paralleled by a disrupted HPA axis activation, since ACTH and cortisol levels were similar among the groups (with exception of a slightly decrease in ACTH in M patients). Furthermore, a progressive diminution in DHEA-s levels was found as disease severity progressed (Co *versus* SEV $p<0.05$). Since DHEA-s levels fall along with aging (Kroboth et al., 1999), an age-adjusted analysis was further performed, revealing the same trend. Such data indicate that DHEA-s decline was linked to the severity of chronic disease. Moreover,