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Chagas' Disease: IgG Isotypes Against Cytoplasmic (CRA) and Flagellar (FRA) Recombinant Repetitive Antigens of *Trypanosoma cruzi* in Chronic Chagasic Patients

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The wide range of clinical Chagas' disease manifestations, of which heart involvement is the most significant, because of its characteristics, frequency and consequences, and lack of treatment and cure, justify research in this area. Specific immunoglobulin G (IgG) antibody subclasses have been associated with human Chagas' disease. Thus, in this study, the profile of IgG subclasses against cytoplasmic (CRA) and flagellar (FRA) recombinant repetitive *T. cruzi*–specific antigens was correlated with cardiac (CARD, n = 33), cardiodigestive (CD, n = 7), and indeterminate (IND, n = 20) forms of Chagas' disease by indirect enzyme-linked immunosorbent assay (ELISA). IgG subclasses were detected in almost all Chagas patients studied. Nevertheless, only specific IgG2 isotype FRA was found with a significant statistical difference in CARD patients when compared to IND patients. This result suggests the potential use of this isotype for prognostic purposes, for monitoring the progression of chronic Chagas' disease, and for predicting the risk of CARD damage. This is important information, as it could help physicians to evaluate and manage the treatment of their patients. However, a follow-up study is necessary to confirm our result. J. Clin. Lab. Anal. 21:271–276, 2007. © 2007 Wiley-Liss, Inc.

Key words: isotypic profile; recombinant antigens; *Trypanosoma cruzi*; Chagas' disease; biological marker

INTRODUCTION

The existence of a wide range of clinical manifestations of the chronic phase of Chagas' disease, including cardiac and digestive complications, apart from the absence of symptomatology, has aroused the interest of various research groups (1–3). This is due to the fact that there is no explanation for the clinical polymorphism of this disease (4).

Classification of cardiac (CARD), digestive, cardiodigestive (CD), and indeterminate (IND) forms of the disease is now carried out using an electrocardiogram and X-rays of the thorax and abdomen, apart from patient anamnesis (5). However, for the investigation of earlier alterations such examinations are inadequate. More sensitive propaedeutic techniques are being used to detect cardiac alterations in the early stages of the disease in individuals with the indeterminate form. However, the data furnished by such techniques are still unable to detect early changes in the digestive system (6).

Castro et al. (7), using propaedeutic techniques in a prospective study, concluded that many individuals with a normal esophagram or with a varying degrees of damage did not develop megaesophagus. However, no prognosis can be made as to which patients will

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develop this condition, and progress to this stage cannot be prevented. Existing noninvasive propaedeutic methods cannot, therefore, identify patients who may go on to develop the various clinical forms of the disease; they can only reveal the existing clinical condition (6).

Little is known about the immune and pathogenic mechanisms involved in the transition from the acute to the chronic stage, or regarding factors that contribute to the development of a particular clinical form (4). Studies have demonstrated that the clinical heterogeneity observed in patients with Chagas' disease is associated with a distinct and complex relationship between parasite and host, with direct involvement of the immune system (1,4).

The identification of target molecules using pure proteins, chemically defined and specific to the parasite has been suggested by various authors as a way of finding out more about the immunopathology of Chagas' disease (3,8). Two antigens with these characteristics were used in this study: cytoplasmatic repetitive antigen (CRA), present in evolved forms of the epimastigote and amastigote of *T. cruzi*, and flagellar repetitive antigen (FRA), present in the epimastigote and trypomastigote forms of the parasite (9,10). The immune response to these antigens has already been studied in mice (11–14), in their diagnostic potential and to monitor posttreatment cure (15–17). However, it is not known which isotypes of immunoglobulin G (IgG) are involved in the immune response generated in patients with Chagas' disease in the presence of these recombinant antigens (Ags-Recs).

This study investigates the humoral immune response in Chagas' disease patients to the Ags-Recs CRA and FRA of *T. cruzi*, and relates this response to the CARD, CD, and IND clinical forms of the disease.

METHODOLOGY

The Ags-Recs CRA and FRA

CRA and FRA antigens were obtained as described by Krieger et al. (9). The genes encoding CRA and FRA were cloned and their expression induced using isopropyl-β-D-thiogalactoside. After centrifugation, the proteins were purified by nickel affinity chromatography according to the supplier's directions (Qiagen, Inc., Valencia, CA). The proteins were analyzed by electrophoresis after silver-staining and periodic acid-Schiff treatment as described by Pereira et al. (12).

Study Population

Chagas' disease patients were selected from the Chagas' Disease Outpatients Clinic of the Clinical Hospital of the Federal University of Pernambuco (HC-UFPE) and the Oswaldo Cruz University Hospital of the State University of Pernambuco (HUOC-UPE), both situated in the city of Recife. Healthy individuals were selected to serve as a control group in the metropolitan region of the city. The classification of the CARD, CD, and IND clinical forms was carried out according to the criteria established by the World Health Organization (WHO) (5). Negative serology for infection by *T. cruzi* in two serum tests, not having received a blood transfusion, and not living in an endemic area were the criteria used for inclusion in the control group.

In accordance with the criteria established above, blood was collected from 60 patients with Chagas' disease, 33 of whom had the CARD form (33–73 years old; 19 females and 14 males), seven had the CD form (30–65 years old; three females and four males), and 20 had the IND form (22–69 years old; 10 females and 10 males). Blood was also collected from 40 healthy individuals, 20 of whom were used as a group of non-Chagasic (NC) individuals (19–31 years old; 10 females and 10 males) to compare with those with the disease. The other 20 were used to establish a cutoff (CO) point.

Ethical Considerations

Both the patients with Chagas' disease and the healthy individuals involved in the experiment participated on a voluntary basis and signed the "Terms of Free and Informed Consent" and filled in a research questionnaire. The way they were included in the experiment and the protocols used were approved by CPqAM/Fiocruz's Research Ethics Committee (Protocol 17/03).

Determination of the Isotype Profile Against CRA or FRA Ags-Recs

ELISA plates (Nunc-Immuno Plates, MaxiSorp, 96 wells, Nalge Nunc International Corporation, Rochester, NY) were sensitized with 100 μL/well of CRA (5 μg/mL) or FRA (2.5 μg/mL) Ags-Recs diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6, blocked, and successively treated with sera diluted 1:100 in phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tw) and, subsequently, with biotin-conjugated immunoglobulins (anti-IgG1, anti-IgG2, anti-IgG3, and anti-IgG4) (Sigma, St. Louis, MO), diluted in PBS-Tw using a kinetic assay. The plates were then incubated with peroxidase-conjugated streptavidin (Pharmingen, San Jose, CA), diluted 1:3,000 in PBS-Tw. The reaction was revealed by the addition of orthophenylenodyamine (0.01%) (Sigma) plus H_2O_2 (0.01%) (VETEC, Rio de Janeiro-RJ, Brazil), diluted in a 0.077 M citrate-phosphate buffer, pH 5.0. The reaction

was measured using the ELISA reader (BIO-RAD 3550; MTX Labsystems, Inc., Vienna, VA) at 490 nm.

The CO was established for each plate, calculating the mean of the optical densities (DOs) of healthy individuals plus two standard deviations (SDs). The results were expressed in the form of a reactivity index, as described by Pereira-Chioccola et al. (18), whereby the values of the means of DOs of the samples were divided by the CO of their respective plates, to enable comparison of the results from different plates.

Statistical Analysis

SPSS version 8 (Statistical Package for Social Sciences; SPSS, Inc., Chicago, IL) was used to analyze the results. The nonparametric Mann-Whitney and Wilcoxon tests were used to evaluate the differences between the indices calculated for each sample. The difference between the results obtained was considered significant for $P<0.05$.

RESULTS

The humoral immune response generated by patients with different clinical forms of Chagas' disease, calculated in terms of response to the CRA or FRA Ags-Recs, showed varying levels of reactivity for each isotype in the case of the different clinical manifestations of Chagas' disease (Tables 1 and 2).

It was observed that, in the case of CRA Ag-Rec, the IgG1, IgG2, and IgG3 isotypes were capable of differentiating some groups of patients with Chagas' disease from the NC individuals, as their reactivity indices were significantly higher than NC individuals. However, there was no significant difference between the reactivity indices for the isotypes analyzed in patients with the CARD, CD, and IND forms of the disease (Table 1). The reactivity indices of the IgG4 isotype were similar in patients with different clinical forms of Chagas' disease and in NC individuals.

In relation to FRA Ag-Rec, the behavior of the IgG1, IgG2, IgG3, and IgG4 isotypes were similar to those found in relation to CRA Ag-Rec. Even when it was possible to differentiate some groups of Chagas' patients from NC individuals, they were not capable of differentiating one clinical form from another (Table 2). Only the IgG2 isotype was capable of differentiating Chagas' disease patients with the CARD form from those with the IND form, as the reactivity indices for the first group of patients were significantly higher than those for the second ($P = 0.010$) (Fig. 1).

TABLE 1. Mean of reactivity indices of IgG isotypes in different clinical forms, compared with the control, against CRA antigen

		Isotypes							
Group	n	IgG1[a]	*P*	IgG2[a]	*P*	IgG3[a]	*P*	IgG4[a]	*P*
CARD	33	3.12	<0.001	1.00	0.048	1.98	0.013	1.14	0.059
CD	7	3.89	<0.001	0.80	0.761	2.22	0.017	0.28	0.077
IND	20	4.69	<0.001	1.44	0.516	1.83	0.083	2.61	0.159
NC	20	0.328		0.691		0.678		0.928	

[a]Mean of reactivity indices.
CARD, cardiac form; CD, cardiodigestive form; IND, indeterminate form; NC, non-Chagasic individuals; *P*, *P*-value comparing clinical form with NC.

DISCUSSION

Investigation into specific immunoglobulins in the serum of individuals with Chagas' disease, together with the use of pure and specific antigens, may be a key combination in elucidating the factors that bring about and maintain the clinical manifestations of the disease (3,8).

In the patients with Chagas' disease selected for this study, the immune responses generated differed according to the subclasses of IgG produced and the clinical forms, when the CRA and FRA Ags-Recs were evaluated. These responses were produced principally by isotypes IgG1 and IgG3, these being able to differentiate most patients with Chagas' disease from the NC individuals. Isotypes IgG2 and IgG4 were also

TABLE 2. Mean of reactivity indices of IgG isotypes in different clinical forms, compared with the control, against FRA antigen

		Isotypes							
Group	n	IgG1[a]	*P*	IgG2[a]	*P*	IgG3[a]	*P*	IgG4[a]	*P*
CARD	33	11.27	<0.001	2.03	<0.001	3.91	<0.001	0.63	0.01
CD	7	10.18	<0.001	2.33	0.011	3.59	0.011	0.40	0.638
IND	20	6.93	<0.001	1.80	0.042	3.42	0.048	0.47	0.542
NC	20	0.346		0.492		1.033		0.761	

[a]Mean of reactivity indices.
CARD, cardiac form; CD, cardiodigestive form; IND, indeterminate form; NC, non-Chagasic individuals; *P*, *P*-value comparing clinical form with NC.

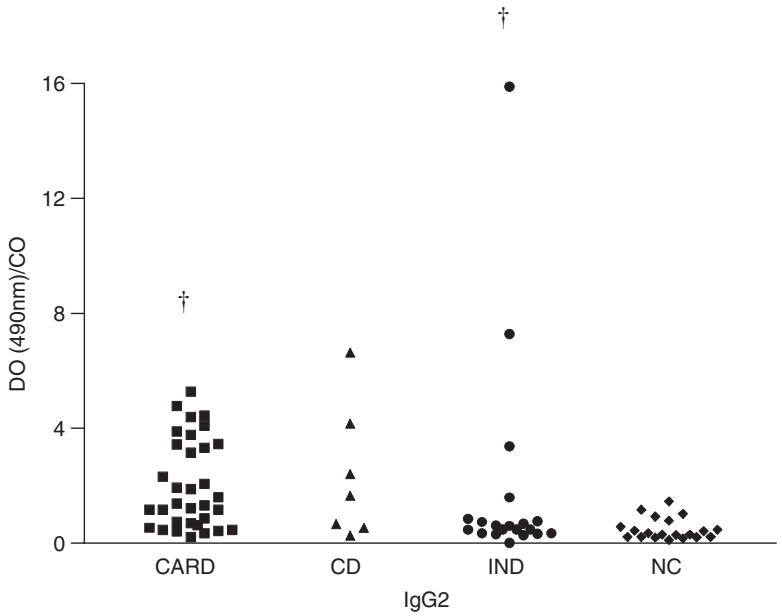

Fig. 1. Reactivity indices of subclasses of IgG2, in patients with chronic Chagas' disease of the CARD, CD, and IND forms, against FRA Ag-Rec. The x-axis shows the reactivity indices, calculated as the mean OD of each sample divided by the CO. The symbols represent the reactivity indices for each sample. †Isotypes that may be differentiated using this isotype ($P = 0.010$). CARD, cardiac form (n = 33); CD, cardiodigestive form (n = 7); IND, indeterminate form (n = 20); and NC, non-Chagasic individuals (n = 20).

present, although to a lesser degree, and with reduced capacity to discriminate between infected and NC individuals.

The diversity of responses found in the case of different clinical forms suggests that immunoglobulins may be related to the clinical condition of individuals with Chagas' disease (3,19). In fact, the immunoglobulins present in infection with Chagas' disease play a variety of roles, both in terms of resistance of the host to the parasite (20,21), and as a cause of tissue damage and changes in the physiology of affected organs (2,22).

The predominance of IgG1 and IgG3 isotypes identified for the CRA and FRA Ags-Recs is no different from the immune humoral responses identified for fixed antigens (23), live antigens (24), and even purified *T. cruzi* antigens (25). It is known that, in the case of live parasites, these isotypes are capable of activating the complement system, thereby contributing to the resistance of the host to infection (20,21,24). It has been shown that the lythic IgG1 isotype was present in high concentrations in patients showing no symptoms of the disease, and thus may be used to predict the risk of cardiac damage in Chagas' disease (24). Another study, carried out by Romeiro et al. (26) also showed that the IgG2 isotype also exhibits lythic activity.

Some studies, using complex *T. cruzi* antigens, have provided evidence of a stronger humoral immune response in patients who show severe forms of the disease compared with those who show no symptoms or who have slight cardiomyopathy (27,28). However, there is no evidence as to which isotypes may be involved in the immunopathology of these severe forms, possibly owing to the complex nature of the antigens used, which prevents detection of variations in the specificities of antibodies for different clinical forms of the disease (3,8). Others (3) have suggested that there is an association between recognition of a specific antigen and the clinical condition of the patient, by virtue of the fact that reactivity against certain recombinant antigens of *T. cruzi* was significantly higher in patients with clinical manifestations of Chagas' disease compared with those showing no symptoms. It can thus be suggested that the use of purified antigens instead of fixed antigens of *T. cruzi*, may reveal interesting isotype responses.

The results of the present study show a diversity of humoral immune responses to the CRA and FRA Ags-Recs. It should be pointed out that the specific IgG2 isotype for the FRA Ag-Rec proved capable of distinguishing patients with the CARD form of Chagas' disease from those with the IND form, as the reactivity indices for this isotype were significantly higher in patients with the CARD form ($P = 0.010$). However the relevance of the presence of this isotype in the immunopathology of Chagasic cardiomyopathy has still not been ascertained.

This marker, once evaluated in a prospective study, may be a further tool in the identification of

asymptomatic Chagas patients who may go on to develop the CARD form of the disease. The physician would then be able to identify the possibility of this development and adapt the patient's course of treatment accordingly.

High levels of this isotype were found in a group of individuals with Chagas' disease from the native American people known as the Toba, in comparison with another group of carriers of the disease from the Mataco community, with the latter group exhibiting much higher frequencies of electrocardiographic abnormalities (8). Other studies have found high levels of this isotype in patients with the CARD and CD forms of the disease, compared with NC individuals (23). However, no studies have shown the capacity to distinguish between Chagas' disease patients with different clinical manifestations, coming from different regions where Chagas' disease is endemic, using either fixed (23), live (24) or purified *T. cruzi* antigens (8,25) by way of the IgG2 isotype. Only one study, carried out by Hernández-Becerril et al. (29), has shown high levels of this isotype to be associated with cardiomegaly. However, it was not established whether it would be possible to monitor the clinical progression of the disease using this isotype, as, in this study, it was not possible to compare the results with those of Chagas' patients with the IND form of the disease.

The specific IgG1 and IgG3 isotypes for CRA or FRA Ags-Recs, which were also selected as potential markers for clinical evolution, presented high levels in all patients with Chagas' disease, irrespective of their clinical condition. As propaedeutic methods currently used to determine the IND form are not particularly sensitive, patients with minor cardiac alterations may be being included in the IND form group (30), the uniform humoral response in the CARD and IND forms making it impossible to differentiate them using these isotypes.

As there are various published studies which show the protective (20,21) and aggressive (2,22,31) functions of antibodies in infection with Chagas' disease, the different isotypes produced during infection in different clinical manifestations may be significant for the immunopathology of the disease (3). Distinguishing patients who have only cardiac alterations from those with both cardiac and digestive alterations would be difficult using the humoral immune response. This may be due to the cardiac component present in both groups of patients. The inclusion of patients with the DIG form of the disease would help to confirm this hypothesis.

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