Antibodies to an Epitope from the Cha Human Autoantigen Are Markers of Chagas’ Disease

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Chagas’ disease is a prevalent disease in South America that is thought to have an autoimmune etiology. We previously identified human Cha as a new autoantigen recognized by chagasic sera. Those sera recognized an epitope spanning amino acids 120 to 129 of Cha, named R3. In the present study we have used the synthetic R3 peptide for the detection of serum immunoglobulin G antibodies from patients at different stages of Chagas’ disease, including a therapeutically treated group. The immunoreactivity with R3 by enzyme-linked immunosorbent assay (ELISA) showed 92.4% sensitivity and 100% specificity for Chagas’ disease sera. This sensitivity and specificity were higher than for any other autoantigen described to date. No anti-R3 antibodies were detected in sera from Leishmania-infected or idiopathic dilated cardiomyopathy patients or healthy controls from the same areas. Moreover, anti-R3 antibody reactivity detected by ELISA correlated with conventional serological tests as indirect immunofluorescence and ELISA assays with Trypanosoma cruzi extracts and other diagnostic tests as indirect hemagglutination. The levels of anti-R3 antibodies increased with progression and symptomatology of Chagas’ disease. More importantly, a statistically significant fall in anti-R3 antibody titer was observed in patients treated with antiparasitic drugs. Those results suggest that the presence of anti-R3 antibodies is a highly specific marker of Chagas’ disease and that R3 ELISA could be helpful in the diagnosis and monitoring of this disease.

Chagas’ disease, which is caused by the protozoan parasite Trypanosoma cruzi, affects several million people in Central and South America (3). Approximately 30% of infected persons develop symptoms of the disease in their lifetime, which include cardiomyopathy, neuropathies, and dilatation of colon or esophagus (27). The finding of a T-cell-rich inflammatory mononuclear cell infiltrate and the scarcity of parasites in heart lesions questioned the direct participation of T. cruzi in chronic Chagas’ cardiomyopathy (CCC) and suggested the possible involvement of autoimmunity (24), although this remains a hotly debated issue (13).

Natural infections occur via the triatomid insect vector and have been almost abolished through vector control programs. Congenital transmission and transfusion of blood from infected donors have become the major routes of transmission of T. cruzi infection often requires a combination of some of the commercially available tests (15). Traditional methods of parasite detection such as xenodiagnosis and hemocultures have low sensitivity and require long periods of time to carry out. Recently, PCR amplification of nuclear or kinetoplast DNA has been shown to be very sensitive (2, 4, 25, 31). However, PCR is not yet feasible for blood bank testing in many of the areas where Chagas’ disease is endemic. At present, the best way of diagnosing an indetermi-
ELISA. ELISA with total T. cruzi antigens was performed in microtiter plates covered with soluble antigens following the directions of the manufacturer (T. cruzi, Biozima-Ch, Polychaco, Argentina). The sera were diluted 1:100. The second antibody was monoclonal anti-human immunoglobulin G (IgG) labeled with horseradish peroxidase. Hydrogen peroxide-tetramethylbenzidine was used for color development, and the reaction was stopped with 2 N H₂SO₄. The developed color was measured in a microplate reader at 495 nm.

An ELISA was developed against peptides R3 and S1. The binding of the peptides to the ELISA microtiter plates (Maxisorp; Nunc) was carried out using 2 to 20 µg/ml of peptide in carbonate buffer (pH 9.6) in a final volume of 50 µl and incubating the plates overnight at 4°C. Blocking was carried out in phosphate-buffered saline (PBS) containing 3% low-fat dry milk and 0.2% Tween-20 for 1 h at room temperature. Sera from chagasic patients were added at 1:100 dilutions in PBS–1% low fat dry milk–0.05% Tween-20 to the plates and then incubated overnight at 4°C. Wells were washed three times with the same buffer and subsequently incubated with goat anti-human IgG (heavy and light chains) horseradish peroxidase-conjugated antibodies (Pierce) for 1 h at room temperature and washed five times. Color development was accomplished by incubation with o-phenylenediamine (Sigma) for 30 min and measured at 450 nm in a microplate reader.

IHA. The IHA was performed essentially as described (18) using commercial kits (Polychaco), and following the instructions of the manufacturer.

RESULTS

R3 ELISA of chronic chagasic sera. A total of 105 serum samples were analyzed. Those included sera from 79 persons in the chronic stage of Chagas’ disease. Figure 1 shows that the majority of chagasic sera, 73 of 79 (92.4%), had IgG against R3 above the cutoff level (OD₄₅₀ of 0.122), with 58 of 79 sera (73%) having ELISA OD values above the cutoff (greater than 1). The specificity of the R3 peptide was analyzed with serum samples from nonchagasic patients, including EHS, and patients with other parasitic diseases such as leishmaniasis or unrelated cardiomyopathy such as IDC. All nonchagasic sera showed OD values below the cutoff level (Fig. 1). The statistical analysis showed that the assay was 92.4% sensitive and 100% specific for chagasic sera, while it showed no sensitivity for the nonchagasic controls (leishmaniasis, IDC, and EHS sera) (Table 1).

Correlation of anti-R3 IgG antibody levels with Chagas’ disease progression. Next, we analyzed the titers of anti-R3 antibodies in sera from patients in different stages of clinical disease. The ELISA reactivity against R3 peptide was compared in serum samples from patients in different stages of Chagas’ disease (11 patients with and 33 patients without symptoms) and 10 serum samples of EHS. Although it was not statistically significant, we observed an increase in the mean anti-R3 titer (from 0.8 to 1.1) with progression of disease, since antibody titer was higher in symptomatic than in asymptomatic chagasic patients (Fig. 2A). We included in the analysis an ELISA based on the S1 peptide, which corresponds to the C-terminal repeats of the shed acute-phase antigen of T. cruzi, a highly immunogenic T. cruzi antigen. The reasons to include it were twofold: shed acute-phase antigen has been considered one of the most immunogenic antigens and the best one for the acute phase, and to compare the R3 peptide ELISA with another peptide-based ELISA (not based on parasite extracts) against which IgG prevalence in samples from chronic patients has been reported (23). Noteworthy, the titers of anti-S1 antibodies were much lower than anti-R3 titers in all patients.

Relationship of anti-R3 IgG antibody levels to treatment of chagasic patients. The titers of antibodies against the R3 peptide and S1 peptide were also tested in a group of 19 asymptomatic chagasic patients treated with nitifurtoxin (Lampit) or benznidazol (Radanil) and compared to 30 sera from untreated patients of the same asymptomatic group. A statistically significant decrease in the mean reactivity against R3 was observed in patients treated with either Lampit or benznidazol (P < 0.01). The analysis of the S1 reactivity, although lower in magnitude, also showed a statistically significant decrease in the OD values after treatment (P < 0.01), (Fig. 2B).

| Table 1. Analysis of reactivity of R3 peptide ELISA with different sera* |
|---------------------------------|--------|--------|--------|
| Serum                          | No. positive/no. tested | Sensitivity (%) | Specificity (%) |
| T. cruzi                       | 73/79  | 92.4   | 100    |
| Leishmaniasis                  | 0/10   | 0      | 100    |
| IDC                            | 0/6    | 0      | 100    |
| EHS                            | 0/10   | 0      | 100    |

* Data from Fig. 1 were analyzed utilizing the area below a ROC curve. The cutoff level was set at an OD₄₅₀ of 0.122. The number of positive samples for individuals infected with T. cruzi or Leishmania, patients suffering IDC, and EHS are indicated. Sensitivities and specificities of the assay are expressed as percentages.
Comparison of R3 peptide ELISA with other serological tests. The results obtained with ELISA of the R3 or S1 peptides were compared with the results of other commercially available diagnostic tests, including IFA, IHA, and ELISA with crude parasite extract, and statistical analyses were performed using the Spearman test. Correlation between the R3 ELISA and IIF (Fig. 3A) and IHA (Fig. 3B) titers as well as with conventional ELISA (Fig. 3C) showed indexes of 0.61, 0.71, and 0.64, respectively ($P < 0.001$). Although the S1 ELISA also showed a direct correlation with IIF, IHA, and ELISA, the indexes were smaller than with the R3 ELISA (0.39, 0.5, and 0.35, respectively, with $P < 0.01$ to 0.001).

**DISCUSSION**

Despite programs of insect control, diagnosis of Chagas’ disease in blood bank screening is necessary nowadays in order to avoid spreading the infection. Currently, the detection of positive samples for *T. cruzi* infection involves a combination of different tests. A recurrent problem is the presence of false-positives due to cross-reactions with sera from patients with other infectious diseases, such as leishmaniasis.

Here, we have tested the potential of the R3 peptide of Cha, a recently described autoantigen (11), as a marker of Chagas’ disease. The presence of specific autoantibodies is used as marker of the disease and of disease progression in several pathologies of autoimmune etiology (7, 8, 29). Here, we report the first description of the usefulness of such a test for the diagnosis of Chagas’ disease. We performed a statistical analysis of the reactivity against the R3 peptide of Cha and found that it is a good marker of the disease. The R3 peptide ELISA showed 92.4% sensitivity and 100% specificity for chronic cha-
gastic sera in comparison with sera from individuals infected with Leishmania and patients suffering IDC and EHS, sera which were below the cutoff value. Although many autoantigens have been described during T. cruzi infection, the percentage of recognition among chagasic sera was lower (6, 9) than for the R3 peptide. The R3 peptide was not recognized by sera from IDC patients, a disease clinically similar to CCC. Very interestingly, this result is contrary to results obtained with other autoantigens which are recognized by both chagasic and IDC sera (5) and indicates the high specificity of the R3 peptide as a marker of Chagas’ disease.

Currently employed serological tests for Chagas’ disease include some commercially available enzyme immunoassays, based on T. cruzi extracts, as well as IIF and IHA assays. These assays are still widely used but often lead to false-positives or -negatives due to subjective interpretation. However, those tests gave between 7.5% and 17.5% positive results for sera from Leishmania-infected patients as well as false-positives and -negatives (15). Many enzyme immunoassays using either crude antigen or recombinant T. cruzi proteins have been recently described with the aim to circumvent this problem (12, 26, 30). R3 is not recognized by sera from IDC patients, a disease clinically similar to CCC. The R3 peptide was not recognized by crude antigen or recombinant T. cruzi infection, usually diagnosed by IHA and IIF, are recognized by the anti-R3 test. Anti-R3 antibody titer tended to increase with disease progression (mean OD increased from 0.8 to 1.1) although the differences were not statistically significant because of the spreading of the OD values (from approximately 0.2 to 1.5). We observed an inverse correlation between nitfurimox/Lampit treatment and anti-R3 antibody titers in asymptomatic patients. This inverse correlation was observed as well with IIF, IHA, and ELISA based on the parasite antigen. Thus, the IgG titers against R3 of positive sera showed a statistically significant decrease in treated patients.

The R3 peptide ELISA showed a stronger titer and correlation with other tests than an ELISA against the S1 peptide, which contains the highly immunogenic C-terminal repeats of shed acute-phase antigen of T. cruzi and is considered a major antigen (23). This result indicated that a stronger reactivity against R3 of Cha than against S1 of shed acute-phase antigen develops during infection. On the other hand, T. cruzi is a polymorphic parasite, and different strains may circulate in different areas. Our results gave similar results when the sera were divided according to their origin (Venezuela and Argentina) (not shown). Another problem that some serologic tests based on T. cruzi antigens present is derived from the cross-reactivity of anti-T. cruzi antibodies with other endemic parasities such as Leishmania (1) and Trypanosoma rangeli (12, 21, 26, 30). R3 is not recognized by sera from Leishmania-infected patients. Although we have not specifically tested reactivity of R3 with sera from T. rangeli-infected individuals, we think this is unlikely by the nature of the test, since these patients have no autoantibodies.

In summary, our results indicate that the detection of anti-bodies against R3 peptide could be used as a confirmation marker of Chagas’ disease. Due to the direct correlation with symptoms and inverse correlation with treatment, it can be used to help monitor the clinical status of patients.

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REFERENCES

19. Paranhos-Bacalla, G., M. Santos, P. Cotrim, A. Rassi, M. Jolivet, M. Ca-