Serological Diagnosis of Chagas Disease with Purified and Defined *Trypanosoma cruzi* Antigens

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Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, widespread in the American Continent. A recent report of the World Health Organization estimated that 16-18 million people are currently infected and that about 90 million individuals living in endemic areas are at risk of contracting *T. cruzi* infection. The high prevalence and continental extension of Chagas disease in Latin America, as well as the constant migration of *T. cruzi* infected individuals to non-endemic areas, require efficient and practical diagnostic procedures (Schmunis 1991,Wendel & Gonzaga 1993).

Chagas disease is characterized by the chronological appearance of specific classes of antibodies during the development of the infection. Antibodies from IgM class first appears as a typical sign of the acute phase of the disease, and there are some reports of an increase of total and specific IgA antibody class at this early phase (Lorca et al. 1995, Umezawa et al. 1996a). Antibodies of the IgG class, already present in the acute phase, accompanies the infection until the chronic phase.

Detection of antibodies against *T. cruzi* antigens by serological methods is still the main support for diagnosis of Chagas disease. The commercial available diagnostic tests are based on the whole or semi-purified antigenic fractions from *T. cruzi* epimastigote (the non-infective forms of the parasite). Considerable variation in the reproducibility and reliability of these tests have been reported by different laboratories, mainly to cross-reactivity with other pathogens and standardization of the reagents (Camargo et al. 1986). Diagnostic tests employing epimastigote antigenic extract have a limited specificity, associated to the fact of they do not possess highly reactive epitopes for IgG/IgM antibodies present in patients with acute or congenital Chagas disease (Umezawa et al. 1996a-c). The epimastigote antigenic fraction is constituted by complex molecules, that favors the appearance of false positive reactions and cross reaction with sera from patients with another infections, mainly visceral leishmaniasis (Chiller et al. 1990). This antigenic heterogeneity does not allow the differential diagnosis between the acute and chronic phases, and also among the clinical manifestations of Chagas disease.

EVALUATION OF RECOMBINANT ANTIGENS FOR CHAGAS DISEASE SERODIAGNOSIS

As discussed above the commercial immunodiagnostic tests are based on the whole or semi-purified extracts of *T. cruzi*. The lack of specific and well characterized antigens prepared under quality-control conditions have introduced a source of variability in the final reagent and controversial results have been obtained with these reagents (Camargo et al. 1986). *T. cruzi* recombinant antigens may provide a convenient tool to improve current methods of serological diagnosis of Chagas disease (Moncayo & Luquetti 1990, Levin et al. 1991, Krieger et al. 1992, Franco da Silveira 1992, Paranhos et al. 1994, Pastini et al. 1994). Cloned peptides carrying well defined epitopes can be produced on a large scale and with acceptable degree of purity. For this reason, we have decided to evaluate the diagnostic efficiencies of different *T. cruzi* recombinant antigens in an ELISA format (Umezawa et al. 1999). Six *T. cruzi* recombinant antigens were expressed in fusion with glutathione S-transferase (GST) in *Escherichia coli* and purified by affinity chromatography. The selected antigens (H49, JL7, B13 and JL8, see Table) are composed by tandem amino acid repeats and showed

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high sensitivity, specificity, and positive and negative predictive values in previous studies (Moncayo & Luquetti 1990, Levin et al. 1991). Two non repetitive antigens A13 (Paranhos et al. 1990) and 1F8 (Gonzalez et al. 1985) were also included in this evaluation. The diagnostic efficiency of the ELISA using the six recombinant antigens was carried out with 541 serum samples (304 samples from chagasic patients, and 237 samples from nonchagasic patients) from nine South and Central America countries (Argentina, Brazil, Bolivia, Chile, Colombia, El Salvador, Guatemala, Honduras and Venezuela). The development and evaluation of the recombinant ELISA was carried out by a central laboratory (E. Umezawa, Instituto de Medicina Tropical da USP, SP, Brasil). The results were compared to those obtained with the whole extract of epimastigote forms.

Four recombinant antigens (1F8, H49, JL7 and B13) showed high sensitivity varying from 93.4% to 99%. A large number of individuals living in endemic areas produce specific antibodies against repetitive amino-acid antigens (H49, JL7 and B13). Interestingly, the sensitivity (99%) and specificity (99.6%) of 1F8 antigen is comparable to that other repetitive antigens indicating that chronic chagasic patients also display antibodies against non repetitive antigens. Serum samples from chagasic patients reacted with, at least, one recombinant antigen, suggesting that a mixture of recombinant antigens may detect anti-*T. cruzi* antibodies in all of serum samples used in this study. The positivity of a hypothetical antigenic mixture composed by the recombinant peptides H49 or JL7, B13 and 1F8 was calculated as being 100%. Available recombinant antigens react with 87-99% of the chronic chagasic sera, suggesting that the combination of two or more antigens to build up a multi-antigen immunoassay may result in a truly reliable *T. cruzi* serodiagnostic test.

In this study the epimastigote antigen showed 100% of sensitivity. However, the specificity of the epimastigote antigenic extract was lower (84%) than recombinant proteins which displayed specificity values between 96.2% (JL8) to 99.6% (A13, B13 and 1F8). The lower specificity of the epimastigote antigen is mainly due to cross-reacting epitopes between *T. cruzi* and *Leishmania* sp. Our results indicate that one of the major advantage of recombinant ELISA for the serodiagnosis of Chagas disease is the lack of cross-reaction with other parasitic diseases such as leishmaniasis. As it has been suggested by several authors (Levin et al. 1991, Krieger et al. 1992, Pastini et al. 1994, Umezawa et al. 1999), the use of a cocktail of recombinant antigens should provide a reliable *T. cruzi* serodiagnostic test. Different complementary antigens could be combined in a relatively simple immunoassay, and lead to the development of a multi-antigen diagnostic kit standardization for routine diagnosis of Chagas disease.

DIFFERENTIAL DIAGNOSIS OF ACUTE, CONGENITAL AND CHRONIC CHAGAS DISEASE BY WESTERN BLOT WITH TRYPOMASTIGOTE EXCRETED/SECRETED ANTIGENS (TESA-BLOT)

As it was discussed above, the diagnosis of acute and congenital Chagas disease, and the follow up of treated chagasic patients are of paramount importance. So far, the unique test described to control the parasitological cure of treated chagasic patients is the lysis of bloodstream trypomastigotes mediated by complement (Krettli et al. 1982). However, several groups point many methodological difficulties for the inclusion of that test in the routine of clinical laboratories (Pereira et al. 1989). For this reason, the search of specific antigens and/or alternative tests remains important for the follow up of the chagasic patients after drug treatment (Almeida et al. 1991, Norris et al. 1994, Krautz et al. 1994, Levy et al. 1996).

TABLE

Characteristics of *Trypanosoma cruzi* recombinant antigens used in this study

Antigen	Repeat (aa)[a]	Size of the insert (bp)[b]	No. of repeats[c]	Size of the fusion protein (kDa)	Function/cellular location	References
H49	68	978	4.5	63.5	Cytoskeleton associated protein	Cotrim et al. 1990
JL7	68	747	3.6	58.0	Cytoskeleton associated protein	Levin et al. 1989
B13	12	600	16.6	51.5	Cytoplasmic/surface antigen	Gruber & Zingales 1993
JL8	14	351	6.7	42.1	Cytoplasmic antigen	Levin et al. 1989
A13	none	755	none	56.0	Cytoplamic antigen	Paranhos et al. 1990
1F8	none	600	none	51.5	Flagellar calcium binding protein	Gonzalez et al. 1985 Godsel et al. 1995

a: number of amino acids (aa) in the repeat; *b*: size of the insert, in base pairs (bp), of the recombinant clone; *c:* number of the repeats in the insert of the recombinant clone.

We have developed an immunodiagnostic test for Chagas disease using the TESA liberated, as exoantigens, in the supernatant of mammalian cell cultures infected with *T. cruzi* (Umezawa et al. 1996c). Exoantigens were collected from supernatants of LLC-MK2 cell cultures, separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters. The test was developed by immunoblotting and termed TESA-blot. The main components of TESA antigens are a 150-160-kDa protein and several components (130-200 kDa) of SAPA antigen (Shed Acute Phase Antigen), the last previously described by Frasch and co-workers (Affranchino et al. 1989). The presence of SAPA/*trans*-sialidase in TESA-blot was confirmed by reactivity with a specific antiserum against SAPA as well as with IgG/IgM antibodies from acute and congenital human sera (Umezawa et al. 1996c).

The evaluation of TESA-blot was carried out with serum samples from 512 patients, 111 non-chagasic including cases of leishmaniasis and other pathologies, and 401 chagasic patients includind acute, congenital and chronic cases. TESA-blot showed 100% of sensitivity and 100% of specificity. Furthermore, this assay allows the discrimination between acute and chronic phases of Chagas disease. We have found that IgGs from chronic chagasic patients reacted with the 150-160 kDa exoantigen whereas IgGs/IgMs from acute patients reacted with the components of SAPA/*trans*-sialidase. This test, until now, have been demonstrated to be useful as a confirmatory test, since it has been used to confirm several inconclusive results obtained by conventional serology. Preliminary results from our laboratory (Matsumoto & Umezawa) showed that a 160 kDa antigen, obtained from DNA recombinant technology, may correspond to the CRP (Complement Regulatory Protein), previously described by Norris et al. (1994). Martins at al. (1985) had previously demonstrated that reactivity with a 160-kDa trypomastigote surface protein could discriminate between cured treated patients from the non cured ones. For these reasons, we started to use these antigens in the follow up of chagasic patients submitted to chemotherapy.

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