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Immunoblotting analyses using two-dimensional gel electrophoresis of *Trypanosoma cruzi* excreted-secreted antigens

Análise de antígenos excretados-secretados de *Trypanosoma cruzi* por *immunoblotting* em gel bidimensional

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ABSTRACT

Trypanosoma cruzi *trypomastigotes excrete-secrete a complex mixture of antigenic molecules. This antigenic mixture denominated trypomastigote excreted-secreted antigens contains a 150-160 kDa band that shows excellent performance in Chagas' disease diagnosis by immunoblotting. The present study partially characterized by two-dimensional gel electrophoresis the immunoreactivity against the 150-160kDa protein using sera samples from chagasic patients in different phases of the disease. Trypomastigote excreted-secreted antigen preparations were subjected to high-resolution two-dimensional (2D) gel electrophoresis followed by immunoblotting with sera from chagasic and non-chagasic patients. The 150-160kDa protein presented four isoforms with isoelectric focusing ranging from 6.2 to 6.7. The four isoforms were recognized by IgM from acute phase and IgG from chronic phase sera of chagasic patients. The 150-160kDa isoform with IF of approximately 6.4 became the immunodominant spot with the progression of the disease. No cross-reactivity was observed with non-chagasic or patients infected with* Leishmania sp. *In this study we provide basic knowledge that supports the validation of trypomastigote excreted-secreted antigens for serological diagnosis of Chagas' disease.*

Key-words: Trypanosoma cruzi. *Excreted-secreted antigens. Two-dimensional gel electrophoresis. Immunoblotting.*

RESUMO

Formas tripomastigotas de Trypanosoma cruzi *excretam/secretam uma complexa mistura de moléculas antigênicas. Essa mistura é chamada* trypomastigote excreted-secreted antigens *e contém uma banda de massa molecular em torno de 150-160kDa que possui excelente performance para diagnóstico de doença de Chagas em* immunoblotting. *No presente estudo foi caracterizado parcialmente, por gel bidimensional, a proteína de 150-160kDa pela análise da reatividade de anticorpos de pacientes chagásicos nas diversas fases da doença. Proteínas do* trypomastigote excreted-secreted antigens *foram separadas por eletroforese de alta resolução em duas dimensões (2D) e submetidas a* immunoblotting *com soros de pacientes chagásicos e não chagásicos. A proteína de 150-160kDa foi identificada em quatro isoformas com pontos isoelétricos variando entre 6,2 a 6,7. As quatro isoformas foram reconhecidas por anticorpos IgM na fase aguda e por anticorpos IgG na fase crônica da doença de Chagas. A isoforma de 150-160kDa, com ponto isoelétrico de aproximadamente 6,4 tornou-se imunodominante dentre as demais com a progressão da doença. Não foi detectada reatividade cruzada com os soros de pacientes não chagásicos ou pacientes infectados com* Leishmania sp. *Os dados obtidos nesse trabalho, reforçam a importância da utilização do* trypomastigote excreted-secreted antigens *para o diagnóstico sorológico da doença de Chagas.*

Palavras-chaves: Trypanosoma cruzi. *Antígenos excretados/secretados. Eletroforese em duas dimensões.* Immunoblotting.

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Chagas' disease caused by *Trypanosoma cruzi*, is still a major health problem in Latin America. Despite the decrease in incidence and prevalence in endemic areas[26 27], the World Health Organization has estimated 16-18 million infected people and approximately 90 million individuals undeniably living at risk of contact with *T. cruzi*[20]. The debilitating morbidity among these infected persons remains a serious obstacle to health and economic development in Latin America, particularly in poor rural areas.

Currently, the procedures available for control of Chagas' disease are limited. There are no vaccines available and curative measures are restricted to two drugs. Chemotherapy is most effective during the acute and early chronic phase of infection, but has several adverse effects and may not always eliminate the parasite[6].

The acute phase of Chagas' disease follows the introduction of infective forms by vector transmission or contaminated blood transfusion. High number of parasites in the bloodstream, fever, adenomegaly and presence of specific IgM antibodies characterize this phase. However, these symptoms are not frequently observed in endemic areas, where the infection usually has a benign clinical course leading to an indeterminate phase[19 24]. Later in life, 15-20% of individuals in this indeterminate asymptomatic phase evolve to a severely incapacitating chronic phase, the triggering factors for this outcome remain unknown[8].

Serological diagnosis of american trypanosomiasis currently relies upon indirect immunofluorescence, indirect hemagglutination and enzyme-linked immunosorbent assay (ELISA). Most of the commercial tests currently available use whole or semi-purified fractions of *T. cruzi* epimastigotes as the source of antigen. Despite a high sensitivity for antibody detection in chronic patients, epimastigote antigens produce false positive reactions and produce cross-reactivity with sera from patients with other infections, mainly leishmaniasis[5]. Thus, several authors have used recombinant antigens for Chagas' disease screening[16 28 29]. However, the variation in sensitivity, specificity, antigenic preparation and standardization of reagents and procedures reported by different laboratories leads to a considerable percentage of unsatisfactory results that need to be confirmed by other methods[3 11]. This is a critical issue for blood banks, as none of the methods available can be considered sufficiently sensitive for an efficient donor screening of Chagas' disease[21].

Trypomastigote forms of the parasite secrete several antigens into the supernatant of infected cells in culture that includes glycosidases[23], glycosyltransferases[4] and proteases[2 13 33]. This mixed group of molecules is recognized by antibodies produced during the acute and chronic phase of Chagas' disease[14 9] and generally is named trypomastigote excreted–secreted antigens (TESA)[30]. When these secreted antigens are used to identify antibody populations present in the serum of chronic patients by immunoblotting, a 150-160 kDa protein is recognized from 100% of chronic chagasic sera and displays no cross-reactivity with other parasitic diseases[21 30]. Recent reports have shown that TESA utilized in ELISA assays result in excellent sensitivity and specificity for Chagas' diagnostic, in both acute and chronic patients[31].

Our aim in this study was to partially characterize the 150-160 kDa component of TESA using high resolution two-dimensional electrophoresis for protein separation and sera from patients in different phases of *T. cruzi* infection as probes. We hope to provide basic knowledge that could support the validation of TESA for serological diagnosis of Chagas' disease.

MATERIAL AND METHODS

Human sera. We collected sera samples from 25 chagasic seropositive patients and 20 non-chagasic seronegative patients from Brazil. Among the selected chagasic patients, ten were in chronic phase, with positive serology for Chagas' disease following the criteria utilized for blood donors screening (indirect immunofluorescence and ELISA). All chronic patients' presented negative results for specific IgM and both tests were positive for IgG; physical examination and electrocardiography were performed for clinical assessment. Early acute patients included one case of accidental laboratory infection and four cases of vectorial transmission. Clinical symptoms, parasitological tests and presence of specific IgM established the presence of acute disease. These five patients had either absent or low levels of specific IgG as detected by conventional serology, which in addition to clinical and epidemiological data, allowed their classification as early acute infection. Ten cases of vectorial transmission presenting with high titers of specific IgM and IgG were classified as late acute infection irrespective of the presence or absence of clinical symptoms. The twenty non-chagasic seronegative patients consisted of ten cases of cutaneous leishmaniasis and ten healthy blood donors included as negative controls. All sera samples were preserved in pH 7.2 buffered glycerol (v/v) and aliquots were stored at -20°C to avoid protein degradation[28]. The research protocol presented in this manuscript and the consent forms for sample collection and utilization were approved by the Committee for Human Research – Federal University of Uberlandia and Hemominas.

Antigen preparation. TESA were obtained essentially as described[30]. Briefly, trypomastigotes of the Y strain were maintained by serial passages in Swiss mice. The blood collected on the seventh day after inoculation was used to infect HEP-2 cell monolayers maintained in DMEM enriched with 10% fetal calf serum (FCS). Following incubation for three days at 37°C in 5% CO_2, the culture was gently rinsed three times with DMEM and the medium was changed to DMEM 1% FCS. The culture supernatants (TESA) were collected on post-infection day 6 (~10^7 parasites.ml^{-1}) then centrifuged at 2800 x*g* for 10 min at 4°C and filtered through a 0.22 µm pore size cellulose acetate membrane. Protease inhibitors cocktail (1 mM PMSF, 0.5 mg.ml^{-1} aprotinin, and 0.5 mg.ml^{-1} leupeptin) was added to TESA and aliquots were stored at -70°C. The specific concentration of TESA in our preparations could not be determined by conventional assays due the content of proteins present in the medium, such as albumin.

One and two-dimension gel electrophoresis. The TESA were prepared for one-dimension SDS-PAGE (1D) by mixing 150µl of culture supernatant with equal volume of SDS-sample buffer (3% sodium dodecyl sulfate, 60mM Tris-HCl [pH

6.8], 5% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue). The samples were boiled for 5 min at 100°C and loaded in a preparative 7% polyacrylamide gel. For two-dimensional gel electrophoresis (2D), the supernatants were diluted (1:3) in isoelectric focusing sample buffer (8M urea, 4% CHAPS, 18mM DTT, 2% Pharmalite 3-10). The first-dimension isoelectric focusing (IEF) was carried out using Immobiline Dry Strip (pH 4-7L, 7cm long, Pharmacia Biotech) in a Multiphor II electrophoresis system (Pharmacia Biotech, New Jersey, USA). The isoelectric focusing was performed at 20°C with the following program: 500V for 1 min, 500 - 3,500V rising gradient over 90 min, and 3,500V for 4h. After completion of the first dimension procedure, the gel strips were incubated in equilibration buffer at room temperature for 15 min (50mM Tris-HCl [pH 6.8], 6M urea, 2% [w/v] SDS, 30% glycerol, 0.01% bromophenol blue and 130mM dithiothreitol (DTT). This was followed by another incubation in equilibration buffer that contained 150mM iodoacetamide instead of DTT. The equilibrated gel strips were placed on top of 7% SDS-polyacrylamide gels and the electrophoresis was conducted at 60V and 10°C.

Immunoblotting. The proteins separated in the 1D and 2D PAGE gels were transferred onto polyvinylidene difluoride membrane (PVDF, millipore). The membranes were blocked with 5% defatted milk in PBS for 2 hours, then incubated overnight at 4°C with patient sera diluted 1:50 in PBS containing 1% defatted milk (PBS-M1%). After washing, the blots were probed with goat anti-human IgG-peroxidase or anti-human IgM-peroxidase (Sigma) in PBS-M1% for 2 hours followed by a cycle of washes. Antigen-antibody reactions were observed after detection of peroxidase activity by H_2O_2 and diaminobenzidine (Sigma).

RESULTS

In our one dimension immunoblotting assays with sera from early acute patients, IgM antibodies consistently recognized TESA proteins in a range of 80-200kDa in a ladder-like pattern (Figure 1a). TESA-blot for specific IgG in these patients showed reactivity to proteins below 100kDa with a weaker overall intensity when compared to IgM assays (Figure 1b).

The patients grouped as late acute phase showed similarities on their TESA-blot pattern for specific IgM (Figure 1c) and IgG (Figure 1d). The 80-200kDa ladder-pattern was present in both assays. Interestingly, most of the patients in this group presented one band with approximately 160kDa, included in the ladder pattern, that showed increased intensity when compared to the others at the same range of molecular weight.

Serological analysis of the chronic patients by one dimension TESA-blot showed an intense IgG reaction at 150-160 kDa in all tested samples (Figure 1f). Some patient samples showed a few bands above this area and most of them recognized bands in the range of 80-120kDa. IgM specific assays with samples from these patients failed to recognize any TESA proteins as shown in Figure 1e.

No antibody reactivity was observed in TESA-blot using samples from healthy blood donors or patients with cutaneous leishmaniasis (data not shown).

Serum samples from early acute infection when analyzed by 2D TESA-blot showed a scattered IgM reaction ranging from pI 5.8 to 6.7 and 80 to 200kDa. The ladder-like pattern observed in 1D TESA-blot revealed a larger number of spots in 2D analysis. IgM reactivity against the 150-160kDa protein was observed with poor definition towards the alkaline side

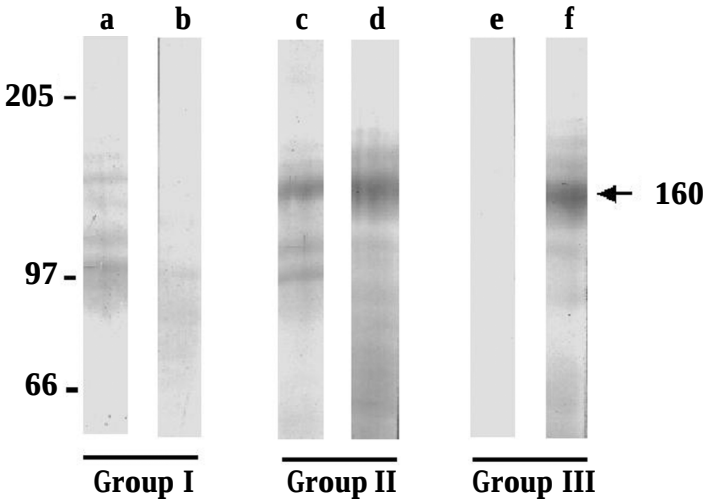

Figure 1 - One-dimension immunoblot of the serum from early acute (Group I), late acute (Group II) and chronic infection (Group III) with Trypanosoma cruzi. *Lanes a, c and e show IgM reactivity and lanes b, d and f show the IgG profile against excreted-secreted antigens. The arrow points to the 160kDa band and molecular weight markers (myosin 205kDa, phosphorylase b 97kDa and albumin bovine 66kDa) are shown on the left. These data are representative of the all sera samples.*

of the blotting, as shown by the arrow heads (Figure 2a). A similar IgM profile was observed in late acute infection (Figure 2b).

The IgG detected in the late acute infection also presented a scattered reaction ranging from pI 5.8 to 6.7 and 70 to 180kDa. However, IgG showed a slightly distinct pattern when compared to IgM. In the late acute infection four defined spots, with pI 6.2-6.7, were recognized by IgG in the 150-160kDa region, as shown by the arrow heads (Figure 2c). Serum samples from chronic patients, when analyzed for specific IgG in 2D immunoblotting showed strong reaction against the four spots described above. Among these dots, a dominant responsiveness was observed against the spot located at pI 6.2 - 6.4 in most of the chronic patient samples (Figure 2d). The frequency of bands recognized in all sample sera were similar (Table 1).

Table 1 - Frequency for recognition in 2D gel of 160 kDa protein, according to chagasic and non-chagasic serum samples.

Serum Samples	Isoforms (pI)			
	5.8 – 6.7		6.2 – 6.7	
	IgG	IgM	IgG	IgM
Early acute phase	0%	100%	-	-
Late acute phase	100%	100%	-	-
Chronic phase	-	NR*	100%	NR
Non-chagasic	NR	NR	NR	NR

*Non reactivity

As a complementary experiment, 2D membranes were probed with polyclonal antibodies generated against a fragment of *T. cruzi* 160kDa recombinant protein (kindly provided by Dr. Eufrozina S. Umezawa, IMT-USP, São Paulo). Our results showed that the four spots located at the range of 150-160kDa and pI 6.2-6.7 were equally immunostained by the 160kDa specific antibody (data not shown).

DISCUSSION

In this study, we grouped the infected patients into early acute, late acute and chronic phases of *T. cruzi* infection. This classification serves for academic purposes and does not necessarily represent the epidemiological scenario of the disease. Approximately 50% of the chagasic population in endemic areas have the indeterminate form of Chagas' disease. The acute phase frequently goes unperceived due to a lack of clinical symptoms, the majority of the infected population is unaware of their health condition[25].

The epidemiological features and peculiarities of the disease in endemic areas represent a challenge for screening healthy blood donors. In Uberlândia, MG, Brazil, a well characterized endemic area, up to 4% of the volunteers for blood donation are rejected due to inconclusive conventional serology for Chagas' disease. This data encouraged us to search for more efficient diagnostic tools, especially in those cases where conventional techniques can not provide a definitive result.

Trypanosoma cruzi as well as other trypanosomatids synthesize and secrete a broad variety of molecules with biological activity during their lifecycle. The fact that *T. cruzi* is transmitted by an insect vector and is able to invade several cell types within the host indicates the requirement of a very versatile adaptive system or systems. The specialized coat of glycoproteins and

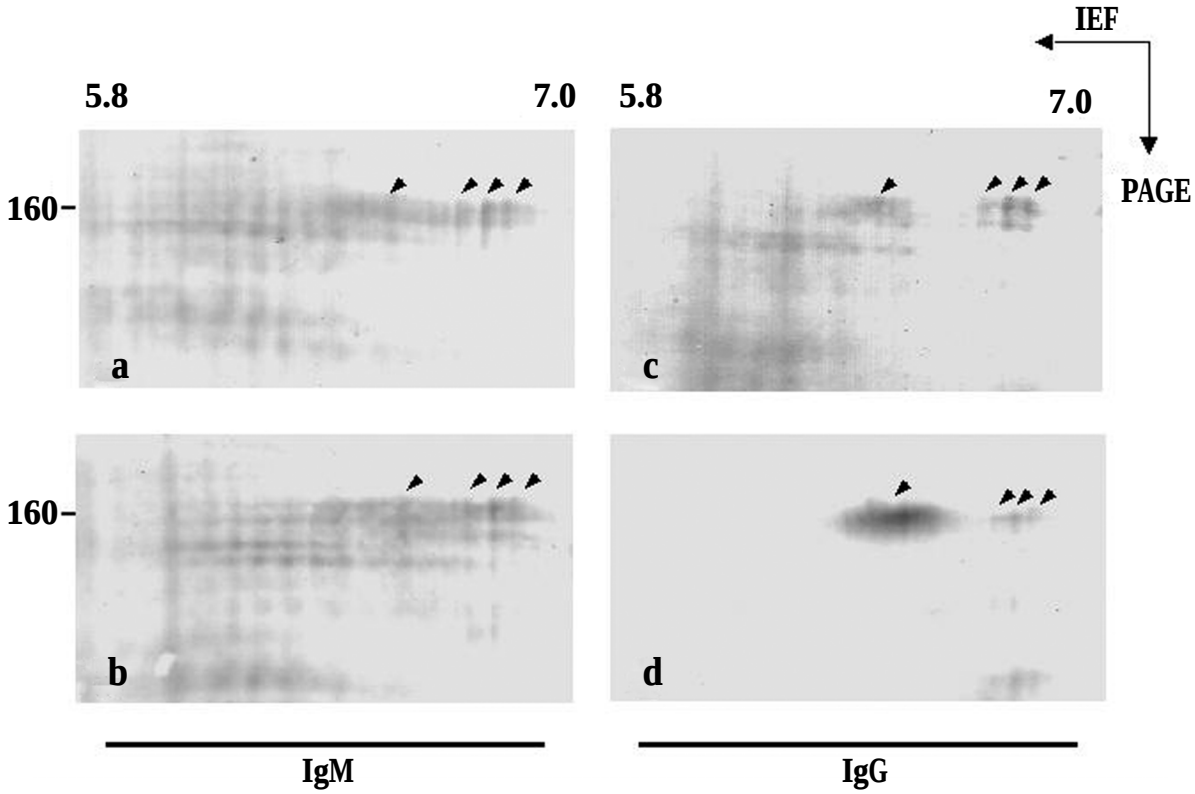

Figure 2 - Two-dimension immunoblot of chagasic patient sera. Figures 2a and 2b show antigens detected by IgM during the early acute and late acute infections respectively. Figures 2c and 2d represent IgG reactivity during the late acute and the chronic infections respectively. The arrowheads point to 160kDa protein isoforms. These data are representative of the all sera samples.

glycolipis on the parasite surface together with secreted proteins all have a crucial role in this environmental transition and adaptation[18].

Many of these secreted molecules belong to the *T. cruzi* transialidase (TS) family[7]. During the early stage of infection an intense shedding of TS molecules (also called shed acute phase antigens [SAPA]), elicits the production of specific antibodies that function as markers of acute infection[1]. Recent publications have supported that transialidase family antigens are associated to cellular invasion, due to their capacity to bind cell receptors and extracellular matrix proteins[10 17]. TS proteins have also been implicated in immune response modulation leading to a down regulation of T cells activity[12]. Later in the disease course, a clear detection of antibodies against the 160kDa secreted protein is considered indicative of chronic Chagas' disease[32].

Following the criteria described above, the pattern of specific IgM produced during the early acute infection suggests that these antibodies are directed against TS repeats contained in SAPA complex[15]. During the early acute phase, most samples contained IgG that weakly recognized antigens below 100kDa. Further investigations will focus on the characterization of this antibody population.

In the late acute phase IgM and IgG revealed strong bands of approximately 150-160kDa. A lower intensity of reaction was observed in other bands above and below the 150-160 kDa range that probably represents antibodies against TS repeats present in SAPA molecules.

As expected, no IgM was detected in the chronic infection and the 160kDa protein became the dominant antigen revealed by the IgG immunoassay.

Two-dimensional analysis of ES antigens immunolabeled by human chagasic serum revealed several spots in the 80-110kDa region with a wide range of isoelectric points (pI between 5.4-6.7)[22]. In the present work, the IgG profile displayed dominant responsiveness against the dot located at 160kDa and pI 6.2-6.4, which was the major immunological target in secreted antigens during the chronic infection (Figure 2d). Five out of ten samples from chronic patients remained reactive to TS repeats in various degrees. These data suggest that TS immune stimuli can remain active through the course of the disease leading to a long-lasting specific antibody production.

The combined analysis of 1D and 2D immunoblots during the early and late acute phase revealed a high level of complexity in the *T. cruzi* excreted-secreted antigen recognition. The ladder-like pattern, usually containing six bands, observed in 1D blots exposed approximately thirty immunoreactive spots when analyzed by 2D blots. This data in addition to the lack of clear definition in the spots' shape indicate a considerable degree of modification in this group of antigens. The uniformity and definition of these spots suggest the presence of a highly repetitive protein motif and may also indicate that this group of antigens undergoes a considerable degree of post-translational modifications.

It is noteworthy that IgM and/or IgG antibodies recognized four dots at 160kDa through all phases of Chagas' disease in all infected patient samples, as observed in 2D analysis (Figure 2, arrowheads). These dots represent isoforms of 160kDa protein, as indicated by specific polyclonal antibody reaction. In contrast to sera from chronically infected patients the 160kDa immunoreaction was not clearly visible in 1D immunoblot during the acute phase (Figure 1a), this is probably due to a massive antibody response against TS repeats that masked the 160kDa band.

No variation in mass was observed between the four isoforms of the 160kDa protein. However, each isoform has a particular isoelectric focusing point, which might be related to modifications, such as phosphorylation that alter the proteins' electric charge.

It is interesting to observe how the immune system shifts the response directed to secreted antigens. The SAPA responsiveness slowly decreased during the progress of disease[14], while 160kDa complex became the major excreted-secreted immunogen.

In summary, we have shown that *T. cruzi* excreted-secreted antigens comprise a complex mixture of antigens, as demonstrated by high-resolution 2D immunoblotting. Our data, although analyzing a small number of patients, correlated with other studies indicating that TESA is a highly efficient tool for Chagas' disease diagnosis in either acute or chronic stages.

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