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# Antigenic analysis of *Trypanosoma cruzi* strains by crossed immunoelectrophoresis: demonstration and isolation of antigens particular to some strains

**M.G. Morgado, M. Van Hoegaerden, and B. Galvão-Castro**

Department of Immunology, WHO Collaborating Centre for Research and Training in Immunology of Parasitic Disease, Fundação Oswaldo Cruz, Rio de Janeiro, Cx. Postal 926, CEP 21040, Brazil

**Abstract.** Antigenic differences among soluble extracts of Y, CL, SF, and Colombian strain epimastigotes of *Trypanosoma cruzi* have been demonstrated by crossed immunoelectrophoresis with an intermediate gel containing the heterologous antiserum. Using crossed immunoelectrophoresis with the homologous antiserum, over 30 precipitin lines could be demonstrated for each strain and, even though marked differences were observed in experimental infections, the strains shared a significant number of antigens. In addition, some strain-particular antigens were isolated using affinity chromatography. These antigens could be valuable in the study of biological, immunological, and pathological characteristics of experimental and natural *T. cruzi* infections.

## Introduction

*Trypanosoma cruzi* is the causative agent of Chagas' disease, which afflicts many people in Central and South America. The euryxenic character of this parasite seems to favour the development of several populations or strains. Indeed, experimental studies of these strains have shown that they differ markedly in their morphobiological and histopathological characteristics (reviewed by Brener 1977 and Andrade and Andrade 1979). Biochemical differences among them have also been demonstrated both by isoenzymic studies (Miles et al. 1977, 1978, 1981; Romanha et al. 1979) and restriction endonuclease mapping of kinetoplast DNA (Morel et al. 1980). Furthermore, the analysis of the antigenic composition has shown that strains (Nussenzweig et al. 1963; Nussenzweig and Goble 1966; Gonzalez-Cappa and Kagan 1969; Ketteridge 1975; Andrade et al. 1981; Araújo and Remington 1981), clones (Bongertz and Dvorak 1983), and evolutive stages of *T. cruzi* (Kloetzel et al. 1975; Santiago et al. 1981; Araújo et al. 1982; Nogueira

*Offprint requests to:* M.G. Morgado

et al. 1982) also differ in expression of both cytoplasmic and surface components.

Despite of the antigenic complexity of *T. cruzi*, comparisons of several strains have been carried out by immunodiffusion and immunoelectrophoresis, which are techniques of poor sensitivity and resolution for the analysis of complex mixtures of antigens (Anders et al. 1982). A preliminary study using crossed immunoelectrophoresis showed a very high level of antigenic complexity, as well as wide antigenic differences among three strains of *T. cruzi* (Morgado et al. 1982). The present work investigated the antigenic profiles of four *T. cruzi* strains, previously characterized by morphobiological and biochemical studies. In addition, some antigens particular to two strains were isolated by affinity chromatography. These antigens were called "strain-particular" instead of "strain-specific" since it is not yet known whether they are expressed in other *T. cruzi* strains.

## Materials and methods

*Parasite strains.* Y (Silva and Nussenzweig 1953), CL (Brener and Chiari 1963), and Colombian (Frederici et al. 1964) strains were initially obtained from Dr. Z. Brener (René Rachou Centre, Belo Horizonte, Brazil), and São Felipe (SF) strain (Andrade 1974) was obtained from Dr. S. Andrade (University of Bahia, Salvador, Brazil). The strains were maintained by serial passages in 20 g, male, outbred Swiss Webstar mice and were characterized by parasitemia, mortality levels, tissue tropism and histological alterations, corroborating previous descriptions (Andrade 1974; Melo and Brener 1978). To obtain the epimastigote forms, hemocultures were made in liver infusion tryptose (LIT) medium and the parasites were maintained at 28° C by serial passages every 2 days.

*Preparation of antigens.* Parasites were harvested on the 2nd day of culture ($10^8$ cells/ml). They were washed 6 times (2000 *g*, 15 min) with 10 mM phosphate-buffered saline (PBS), pH 7.2. The pellet containing approximately $10^{11}$ organisms was resuspendend in 15 ml PBS, sonicated 5 times (3 min each) at 95 W in an ice bath (Branson Sonifer Gel Disruptor B-15), and ultracentrifuged for 30 min at 100000 *g*. The supernatant was filtered through a 0.2 μm Millipore membrane and stored at −70° C. Protein concentrations were determined according to Lowry et al. (1951), using bovine serum albumin as a standard.

*Immunization of rabbits.* New Zealand rabbits were immunized subcutaneously with 1.5 mg protein of the soluble extract emulsified in Freund's complete adjuvant. Forty days afterwards, they received a booster with the same quantity of protein emulsified in Freund's incomplete adjuvant (FIA). The quantity was increased to 5 mg, also emulsified in FIA, for the third (day 48) and fourth (day 78) injections. Blood was collected from the marginal ear vein on days 5, 7, and 9 after the second and third boosters and allowed to clot for 1 h at room temperature and overnight at 4° C. The serum was separated and centrifuged at 1500 *g*, 10 min. All antisera were analysed by immunodiffusion (Ouchterlony 1958) and immunoelectrophoresis (Grabar and Williams 1953) against their homologous antigens, pooled, and stored at −20° C.

*Crossed immunoelectrophoresis (CIEP) (according to Clarke and Freeman 1968).* Briefly, glass plates of 6 × 9 cm were covered with 1.2% w/v agarose gel (Behring) in Tris-barbital buffer, pH 8.6, ionic strength 0.02. The antigen (250 μg of protein) was run in the first dimension (10 V/cm, 45 min, 4° C). Electrophoresis in the second dimension was carried out at 4° C, applying 2 V/cm for 18 h through a gel containing concentration of 20 to 54 μl/cm$^2$ of the homologous or heterologous antiserum, according to their antibody titres (Table 1). After washing and drying, the plates were stained for 10 min in 0.5% (w/v) Coomassie brilliant blue R-250 in ethanol-glacial acetic acid-water (45:10:45) and destained in the same solvent.

**Table 1.** Number of anodic (A) and cathodic (C) precipitin lines observed by CIEP analysis of extracts of Y, SF, CL, and Colombian strains with homologous and heterologous antisera

| Antiserum | | Antigens | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Type | Concentration ($\mu l/cm^2$) | Y | | SF | | CL | | Colombian | |
| | | A | C | A | C | A | C | A | C |
| Anti-Y | 20 | 35 | 4 | 28 | 3 | 35 | 2 | 30 | 3 |
| Anti-SF | 53.5 | 30 | 3 | 29 | 4 | 28 | 3 | 27 | 4 |
| Anti-CL | 20 | 31 | 0 | 30 | 0 | 36 | 0 | 30 | 0 |
| Anti-Colombian | 33.3 | 29 | 3 | 30 | 4 | 28 | 3 | 28 | 3 |

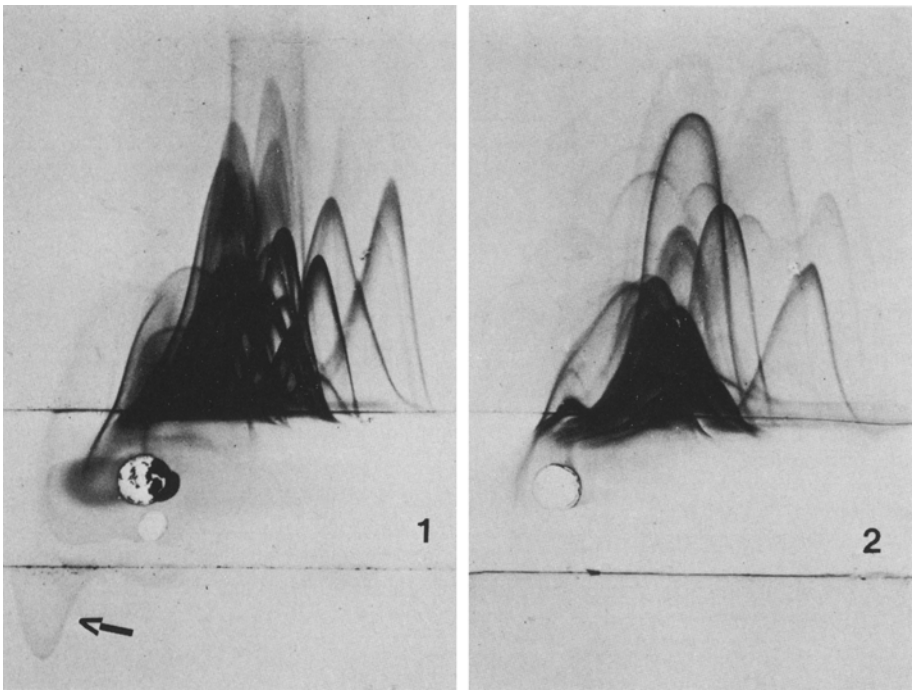
**Table 2.** Antigens particular to Y, SF, CL, or Colombian strains of *T. cruzi* observed by CIEP with the heterologous antiserum in the intermediate gel and the homologous antiserum in the upper gel

| Antiserum in the intermediate gel | Number of precipitin lines observed in the upper gel | | | |
|---|---|---|---|---|
| | Antigens | | | |
| | Y | SF | CL | Colombian |
| Anti-Y | 0 ($83.3\ \mu l/cm^2$)[a] | 1 ($83.3\ \mu l/cm^2$) | 3 ($57.1\ \mu l/cm^2$) | 1 ($83.3\ \mu l/cm^2$) |
| Anti-SF | 2 ($66.6\ \mu l/cm^2$) | 0 ($83.3\ \mu l/cm^2$) | 4 ($57.1\ \mu l/cm^2$) | 2 ($71.4\ \mu l/cm^2$) |
| Anti-CL | 0 ($57.1\ \mu l/cm^2$) | 0 ($83.3\ \mu l/cm^2$) | 0 ($83.3\ \mu l/cm^2$) | 0 ($71.4\ \mu l/cm^2$) |
| Anti-Colombian | 2 ($66.6\ \mu l/cm^2$) | 1 ($83.3\ \mu l/cm^2$) | 5 ($83.3\ \mu l/cm^2$) | 0 ($83.3\ \mu l/cm^2$) |

[a] Concentration of the homologous antiserum in the upper gels

*Crossed immunoelectrophoresis with intermediate gel (Axelsen 1973).* Electrophoresis in the first dimension was similar to CIEP. In the second dimension, a slab of gel (6 × 3 cm) containing 83 $\mu l/cm^2$ of a heterologous antiserum was poured along the first-dimension electrophoresis gel. Separated by a slab of gel (6 × 1 cm) without antiserum, the upper gel (6 × 3.5 cm) containing variable quantities of the homologous antiserum (Table 2) was poured onto the plate. This method allows a direct comparative analysis of two or more antisera against a single soluble extract each time: the antigens shared by the two strains analysed are precipitated in the intermediate gel, and the specific ones are trapped by the antiserum contained in the upper gel. Electrophoresis conditions in the second dimension were 2 V/cm, 18 h at 4° C.

*Affinity chromatography.* Hyperimmune sera obtained against each *T. cruzi* strain were precipitated at 40% ammonium sulphate saturation. Forty milligrams of these immunoglobulins (Ig) was covalently coupled to 7 ml of CNBr-activated Sepharose 4 B, according to the manufacturers instructions (Pharmacia Fine Chemicals, Uppsala, Sweden, 1976). Samples containing 1 mg of soluble extract of *T. cruzi* Y or CL strains were poured into a 10-ml syringe filled with 7 ml of heterologous Ig-conjugated Sepharose and left at room temperature for 90 min. The unbound material was washed off with PBS, concentrated to the original volume (0.5 ml), and tested against homologous and heterologous antisera by radio-rocket immunoelectrophoresis.




**Figs. 1 and 2.** CIEP showing difference in patterns of precipitin lines between *T. cruzi* soluble extracts and their homologous antisera

**Fig. 1.** First dimension: 250 μg Y strain soluble extract. Second dimension: 20 μl/cm$^2$ anti-Y strain serum

**Fig. 2.** First dimension: 250 μg CL strain soluble extract. Second dimension: 20 μl/cm$^2$ anti-CL strain serum. *Arrow:* cathodic lines in the Y strain extract

*Radio-rocket immunoelectrophoresis* (R-RIEP). Rocket immunoelectrophoresis was performed according to Laurell (1966). Electrophoresis was carried out for 3 h at 10 V/cm and 4° C. The plates were then extensively washed with PBS and incubated overnight in a moist chamber with $8 \times 10^6$ cpm of [$^{125}$I]-protein A in PBS containing 1% BSA at room temperature (Stevens et al. 1981). Protein A was iodinated using the IODO-GEN method according to Markwell and Fox (1978). After incubation, unbound [$^{125}$I]-protein A was removed by several washings with PBS, and the plates were dried and autoradiographed using an intensifying screen (Kodak X-Omatic, regular).

## Results

### *1. Antigenic analysis of the strains*

Crossed immunoelectrophoresis of the soluble extracts of epimastigotes with their homologous antisera was used to determine the antigenic patterns of Y, CL, SF, and Colombian *T. cruzi* strains. It was possible to demonstrate the existence of 39 precipitin lines for the Y strain (35 anodic and 4 cathodic; Fig. 1), 36 for CL (all of them anodic; Fig. 2), 33 for SF (29 anodic and 4 cathodic), and 31 for the Colombian strain (28 anodic and 3 cathodic).

An important antigenic similarity among the strains was revealed by the pattern of reaction of their soluble extracts with heterologous antisera by CIEP. However, differences in the number and height of peaks were apparent when homologous and heterologous precipitation profiles were compared. Moreover, cathodic precipitin lines could not be observed when the CL strain antigen was tested against its homologous antiserum, although 2 or 3 weak precipitin lines appeared in the heterologous reactions. The results of the homologous and the heterologous reactions are summarized in Table 1.

Controls consisted of tests in which culture medium (LIT) was substituted for the antigens. In addition, the antigens were tested by immunoelectrophoresis and immunodiffusion against rabbit anti-normal bovine serum, an important component of the culture medium. In all cases, no precipitin lines could be seen.

To demonstrate directly antigenic differences among the strains, we analyzed the extracts by a CIEP modified by the inclusion of an intermediate gel containing heterologous antiserum. Using this method, some strain-particular antigens with anodic migration were identified. Table 2 shows that Y-strain antigens immunoabsorbed when crossing an intermediate gel containing either anti-SF or anti-Colombian strain sera, still led to two precipitin lines when it reacted with the homologous antiserum in the upper gel. In the same way, the SF strain had one antigen not absorbed by the anti-Y or anti-Colombian strain sera diluted in the intermediate gel, and the Colombian strain has one or two antigens not absorbed by the anti-Y or the anti-SF serum respectively. The CL strain had the greatest number of particular antigenic components, giving at least 3, 4, and 5 precipitin lines with the homologous antiserum when absorbed using anti-Y, anti-SF, or anti-Colombian serum respectively, in the intermediate gel. Figure 3 shows an example of this kind of reaction.

No antigen particular to Y, SF, or Colombian strain could be demonstrated when the anti-CL serum was included in the intermediate gel. Strain-particular cathodic antigens were not detected even when the sensitivity of the method was increased by incubation with $^{125}$I-protein A.

### *2. Separation of "strain-particular" from public antigens*

To isolate antigens particular to the CL or Y strain we used affinity chromatography columns prepared with the different heterologous antibody preparations. For the separation of antigens particular to the CL strain, aliquots of CL extract were passed either through anti-Colombian, anti-SF, or anti-Y columns. The non-adsorbed material was collected and concentrated to the original volume. Each fraction was tested by R-RIEP against the homologous antiserum and the heterologous antisera used in the adsorption procedure. For example, soluble CL antigens, after passing through an anti-Colombian Ig column, still formed one strong rocket line when tested with anti-CL serum and one very faint line with the anti-Y serum. No reaction was observed when this fraction was tested against anti-Colombian or anti-

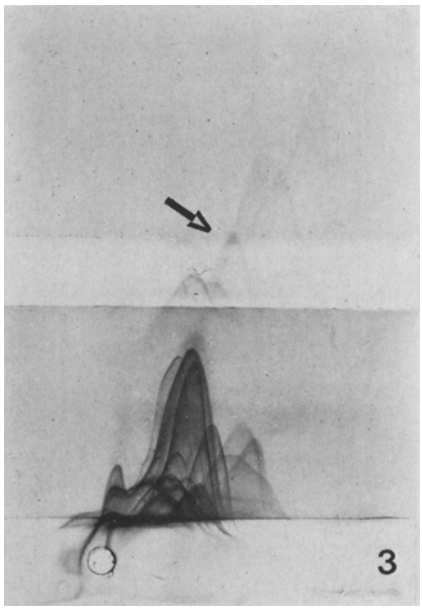



**Fig. 3.** Analysis of the reaction between the CL strain extract with its homologous antiserum after absorption with the anti-SF strain serum on intermediate gel. First dimension: 250 µg CL strain soluble extract. Intermediate gel: 83.3 µl/cm$^2$ anti-SF strain serum. Upper gel: 57.1 µl/cm$^2$ anti-CL strain serum. *Arrow:* localization of antigens particular to the CL strain compared to the SF strain

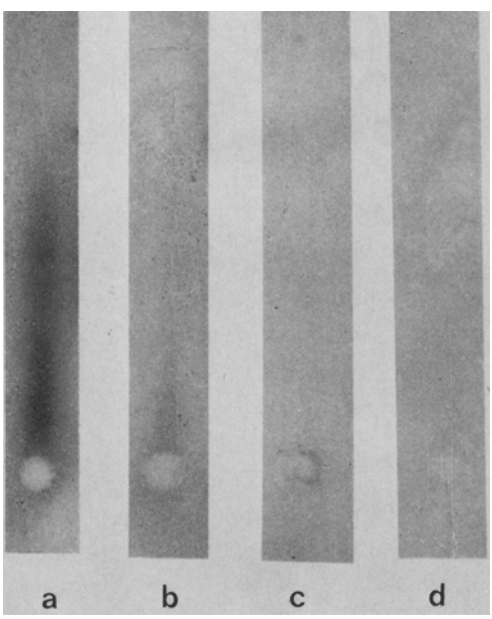



**Fig. 4.** R-RIEP of CL strain-particular antigens obtained after absorption of anti-Colombian strain Ig bound to Sepharose and tested with anti-CL (*a*), anti-Y (*b*), anti-SF (*c*), and anti-Colombian strain sera (*d*)

SF sera (Fig. 4). Similar results were obtained when the CL soluble extract was passed through an anti-SF Ig column, while the CL antigens obtained from the column with anti-Y Ig reacted only with the anti-CL strain serum.

Using the same method, the antigens particular to Y strain were also studied. Y antigens obtained after absorption on columns coupled to anti-SF

**Table 3.** Radio-RIEP of antigens particular to CL and/or Y strains of *Trypanosoma cruzi* obtained by affinity chromatography

| Antigens | Column activity | Antisera used in IEP | | | |
|---|---|---|---|---|---|
| | | Anti-Y | Anti-SF | Anti-Colombian | Anti-CL |
| CL | Anti-Y[a] | 0 | 0 | 0 | 1[b] |
| | Anti-SF | 1 | 0 | 0 | 1 |
| | Anti-Colombian | 1 | 0 | 0 | 1 |
| Y | Anti-SF | 2 | 0 | 0 | 2 |
| | Anti-Colombian | 2 | 0 | 0 | 2 |
| | Anti-CL | 0 | 0 | 0 | 0 |

[a] Specificity of rabbit antiserum (immunoglobulin fraction) coupled to Sepharose 4B
[b] Number of precipitin lines

or anti-Colombian Ig formed two precipitation lines with both anti-Y and anti-CL antisera. No reaction was observed with either the antiserum used in the absorption or with the normal rabbit serum used as control. The fraction of the Y strain antigen that was not specifically adsorbed to the gel coupled to anti-CL Ig did not show any precipitin line with any of the antisera. These results are summarized in Table 3 and indicate that Y and CL epimastigotes have "particular" antigens when compared to both SF or Colombian strains and that the CL strain also has some antigens not found in the other strains studied in this work. This agrees with the results obtained by CIEP with intermediate gel.

## Discussion

In this study we were able to identify important antigenic differences between Y, CL, SF, and Colombian strains of *T. cruzi,* which are also distinguished by morphobiological, biochemical, and histopathological characteristics. In fact, the Y and CL strains, for example, have been named "polar strains" (Brener 1977), since they differ in the morphology of bloodstream forms, parasitemia levels, distribution of parasites in the tissues of the murine host, growth and differentiation in cellular or acellular media, and reaction with specific antibodies.

Although the existence of antigenic differences between strains of *T. cruzi* from diverse geographical areas or host sources is well known (Nussenzweig et al. 1963; Nussenzweig and Goble 1966; Gonzalez-Cappa and Kagan 1969; Ketteridge 1975; Andrade et al. 1981), the relatively low resolution of the techniques employed so far has limited the identification of the antigenic components of these parasites. Thus, a much higher antigenic complexity than had been demonstrated so far was observed in the present study, where CIEP with and without an intermediate gel was used. In addition, we succeeded in isolating some antigens particular to Y and/or CL strains by affinity chromatography. These antigens were called "strain-par-

ticular" instead of "strain-specific" since it is not yet known whether they are expressed in other *T. cruzi* strains not analysed in this work.

On the other hand, the presence of a large number of antigens shared by these four strains was clearly demonstrated by the patterns of cross reactivity of each soluble extract with the heterologous antisera. Some of these common antigens might be implicated in the cross-protection mechanism already observed among *T. cruzi* strains (Norman and Kagan 1960; Gonzalez-Cappa et al. 1974; McHardy and Elphick 1978).

Although the CIEP with intermediate gel is a highly sensitive method for the analysis of complex antigen mixtures, we should take into account that this technique reflects the potency and multispecificity of the antisera used. However, in a preliminary study (data not shown) using antibodies produced by hybridoma technology from mice immunized with CL extract, monoclonal antibodies specific for CL, when compared with Y strain epimastigotes, were demonstrated by indirect immunofluorescence. Also, the analysis of the four soluble extracts of Y, CL, SF, and Colombian strains, using sodium dodecylsulphate polyacrylamide gel electrophoresis, showed different patterns of protein components, thus reinforcing the hypothesis of antigenic dissimilarities among *T. cruzi* strains. Thus, these antigenic differences, taken together with the variations in biochemical and biological characteristics, especially those related to host-parasite relationship, should improve the understanding of the intra-specific variations in this species and its phylogenetic relationship with other trypanosomatides.

We do not yet know whether these strain-particular antigens are important in the pleomorphism of Chagas' disease and in the variations observed in experimental host-parasite relationship, or if they are expressed during the whole evolutive cycle of the parasite. However, antigenic differences among bloodstream trypomastigotes of several *T. cruzi* strains have also been reported (Krettli and Brener 1976; Kloetzel and Camargo 1976; Ferrioli Filho and Oliveira 1982). In addition, Okanla et al. (1982) verified by CIEP that 70% of the antigens present in epimastigotes of Tulahuén strain are also shared by bloodstream and metacyclic trypomastigotes of this parasite. These results may suggest that the strain-particular antigens, observed in epimastigote forms, could also be expressed throughout the evolutive cycle.

On the other hand, it must still be established as to whether some strain-particular antigens are expressed as a result of long-term culture procedures, and do not, therefore, reflect real antigenic differences of the parasites found in natural infections. In fact, although *T. cruzi* (Tehuantepec strain) has displayed antigenic stability when maintained for 5 years in culture medium (Afchain et al. 1979), artificial methods of parasite maintenance may act as a selective factor of populations. For example, Romanha et al. (1979) described differences in isoenzymic pattern obtained from the same strain maintained during different periods of time in culture. Further K-DNA profiles among epimastigotes, all classified as Y strain, but obtained from several laboratories, have been reported to be heterogenous (Morel et al. 1980). However, these variations do not necessarily reflect modifications in the antigenic constitution of the parasites.

Finally, the isolation of large amounts of these "strain-particular" antigens will be necessary for their biochemical characterization and the determination of their possible biological role in the course of Chagas' disease.

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