# High expression of a functional cruzipain by a non-infective and non-pathogenic *Trypanosoma cruzi* clone

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SUMMARY

We compared a *Trypanosoma cruzi* clone unable to infect or induce pathology in mice (CL-14), with virulent *T. cruzi* (Y and CL strains) in terms of cruzipain expression, subcellular distribution and functional activity. Our results showed that (1) intracellular Y amastigotes expressed R1 (carboxy-terminal) and R2 (catalytic) domains concentrated in cytoplasmic vesicles, while CL-14 presented R1 labelling on membrane clusters and R2 in intracellular compartments, (2) CL-14-trypomastigotes presented R1 and R2 staining preferentially on flagellar and cellular membranes, similar to CL, but different from Y strain intracellular labelling pattern, (3) flow-cytometry revealed higher expression of R1 by CL-14-trypomastigotes than virulent strains, but R2 staining similar to CL-trypomastigotes, (4) CL-14-trypomastigotes presented normal cruzipain activity in gelatin gels, but different banding patterns were found in CL-14 *versus* CL and Y strains. Our data rule out failure in cruzipain expression, activity or subcellular distribution as an explanation for CL-14 biological behaviour, but suggest the expression of a different isoform. These results also cast doubt on the putative role of cruzipain as a target of immunopathological responses, since high levels of functional cruzipain are expressed by a non-pathogenic *T. cruzi*.

Key words: *Trypanosoma cruzi*, cruzipain, cysteine proteinase, infectivity, pathogenicity.

## INTRODUCTION

*Trypanosoma cruzi* expresses a major cysteine proteinase named cruzipain, an enzyme involved in cellular invasion, intracellular multiplication and parasite differentiation (Meirelles *et al*. 1992; Harth *et al*. 1993; Franke-de-Cazzulo *et al*. 1994). Cruzipain is highly immunogenic in human infections (Scharfstein *et al*. 1983; Gazzinelli *et al*. 1990; Martinez *et al*. 1991, 1993; Arnholdt *et al*. 1993) attracting attention to its possible role as a target of protective or pathological immune responses. Circumstantial evidence supports this latter hypothesis, since T cell clones reactive against homologous human lysosomal cathepsin could be obtained from chagasic individuals (De Araújo *et al*. 1996), and depots of cruzipain are found among mononuclear cells that infiltrate myocardial muscle in these patients (Morrot *et al*. 1997). In addition, cruzipain induces plasma leakage from post-capillary venules (Svensjo *et al*. 1997), suggesting a role for the enzyme as a pro-inflammatory factor.

*T. cruzi* CL strain, isolated by Brener & Chiari (1963) is very infective, being able to invade almost all organs and tissues of the host (Lenzi *et al*. 1996). CL-14, a clone isolated from CL strain (Chiari, 1981), is unable to induce infection in adult (C3H, BALB/c or C57BL/6) or newborn mice (Lima *et al*. 1991, Lima, Lenzi & Gattass, 1995), although it is capable of cellular infection *in vitro* (unpublished observations). Mice injected with culture or insect derived trypomastigotes from clone CL-14 lack any of the histopathological lesions characteristic of acutely or chronically infected mice, and exhibit a strong protective immunity that completely prevents the development of parasitaemia in mice further challenged with virulent *T. cruzi* (Lima *et al*. 1991, 1995).

The reason why clone CL-14 does not produce infection or pathology, but nevertheless generates efficient protective immunity against *T. cruzi*, is unknown. Herein, we sought to investigate whether failure in cruzipain expression, distribution or functional activity underlies the biological behaviour of clone CL-14.

## MATERIALS AND METHODS

### *Parasites*

Trypomastigote forms of Y and CL strains were obtained from the supernatant of LLCMK2 (ATCC, Rockville, MD/USA) cell culture infected with bloodstream trypomastigote forms, as previously described (Carvalho & De Souza, 1983), while infected LLCMK2 cell cultures were used in electron microscopy analysis as a source of intracellular amastigotes. Trypomastigotes from CL-

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14, a clone isolated from CL strain (Chiari, 1981), were obtained by *in vitro* metacyclogenesis, performed as previously described (Lima *et al*. 1991). These trypomastigotes were used to infect LLCMK2 cells, and used as a supply of tissue culture trypomastigotes and amastigotes, as described above.

### *Antigen and sera*

Polyclonal antisera against cysteine proteinase was a gift from Dr J. J. Cazzulo (Universidad Nacional de General San Martin, San Martin, Argentina). The presence of specific antibodies was detected by the method of Ouchterlony using the purified enzyme as antigen (Campetella, Martinez & Cazzulo, 1990). Polyclonal antibodies against the C-terminal and catalytic domain of cruzipain were used (Martinez *et al*. 1993).

### *Immunocytochemical localization of cruzipain*

Tissue culture trypomastigotes from clone CL-14, Y and CL strains obtained from the supernatant of LLCMK2 cells by centrifugation (2000 ***g*** for 10 min at 4 °C) were washed twice with phosphate-buffered saline (PBS) and fixed for 60 min in a solution containing 0·1 % glutaraldehyde, 4 % formaldehyde (freshly prepared from paraformaldehyde) in 0·1 M cacodylate buffer, pH 7·2, containing 0·2 % picric acid and 3·7 % sucrose (Sigma, St Louis, MO, USA). Host-cells at day 4 post-infection were rinsed in PBS, fixed as described above, and then gently scraped off with a rubber policeman. After fixation, samples were washed twice with PBS, dehydrated in methanol and embedded in Lowicryl K4M at −20 °C. Thin sections were collected in 400-mesh nickel grids and incubated subsequently for 30 min at room temperature in PBS, pH 8·0, containing 1·5 % bovine serum albumin (BSA) and 0·01 % Tween 20 (PBS–BSA–Tw). Then they were incubated in the same solution containing either the polyclonal antibody against the COOH-terminal (anti-R1) or the catalytic (anti-R2) portion of the *Trypanosoma cruzi* cysteine proteinase (diluted 1:300) for 60 min at room temperature. After incubation, the grids were washed 3 times with PBS–BSA–Tw and incubated for 60 min at room temperature in PBS–BSA–Tw containing gold-labelled protein A (10 nm) diluted 1:40. Control grids were incubated in the presence of non-immune rabbit serum or only in the presence of gold-labelled protein A. In order to compare the intensity of labelling, grids containing the different samples were incubated at the same time using the same dilution of anti-cysteine proteinase antibodies and protein-A gold complex. After incubation, grids were washed in distilled water, stained with uranyl acetate and lead citrate and observed under a Zeiss EM-900 (Oberkochen/Germany) transmission electron microscope.

### *Flow cytometry*

Cells of each sample were fixed prior to staining in 2 % formaldehyde–PBS, pH 7·2, for 10 min at 4 °C, washed in PBS and incubated for 15 min in PBS containing 50 mM $NH_4Cl$. After another wash in PBS containing 1 % BSA and 0·02 % $NaN_3$ (PBS–BSA), pH 7·2, cells were counted, separated in aliquots of $5 \times 10^5$ and incubated for 30 min in normal goat serum (1 % in PBS), to block immunoglobulin receptors on the parasite surface. To evaluate cruzipain expression on the parasites, cells were incubated with 50 $\mu$l of the R1 or R2 antibodies (diluted 1:300 in PBS–BSA) and revealed with goat anti-rabbit conjugated to FITC (GAR–FITC, diluted 1:200 in PBS–BSA). Labelling controls were prepared by incubating previously blocked preparations with normal rabbit serum or PBS–BSA followed by GAR–FITC. Cells were then washed twice with PBS, resuspended in 0·5 ml of PBS–BSA and observed under a fluorescence microscope to confirm absence of agglutination. Samples were kept in the dark until analysis. Flow cytometric data were acquired using an EPICS-ELITE (Coulter Eletronics, Florida, USA).

### *Proteinase activity*

Detection of proteinase activity of trypomastigotes lysates was performed in 10 % resolving SDS–acrylamide gels containing 0·1 % copolymerized gelatin as substrate as previously described (Bonaldo *et al*. 1991). Briefly, $10^8$ parasites were submitted to 10 freeze and thaw cycles before solubilization in 100 $\mu$l of sample buffer without 2-mercaptoethanol (2-ME). After electrophoresis, gels were incubated for 1 h in 2 % Triton X–100 in 0·1 M citrate–phosphate buffer, pH 5·5, containing 10 mM 2-ME and for another 1 h in 0·05 % Triton X–100 in the same buffer. Next, the gel was submitted to 3 washes and incubated overnight, at 37 °C, in the citrate–phosphate buffer without Triton X–100. Proteinase specific activities were characterized by adding class-specific enzyme inhibitors to the post-electrophoretic buffers solutions. After staining with 0·125 % Coomassie blue R-250, proteinase activity appeared as clear bands against a blue background.

## RESULTS

### *Immunocytochemical labelling of cruzipain*

The following evolutive forms of *T. cruzi* were analysed: (1) typical trypomastigote forms obtained from the supernatant of LLCMK2 cells and identified by the morphology of the kinetoplast DNA network; (2) intracellular amastigotes morphologically characterized by their round or oval shape and by the structure of the kinetoplast DNA network, which appears as a slightly concave disk formed by a filamentous material arranged in a

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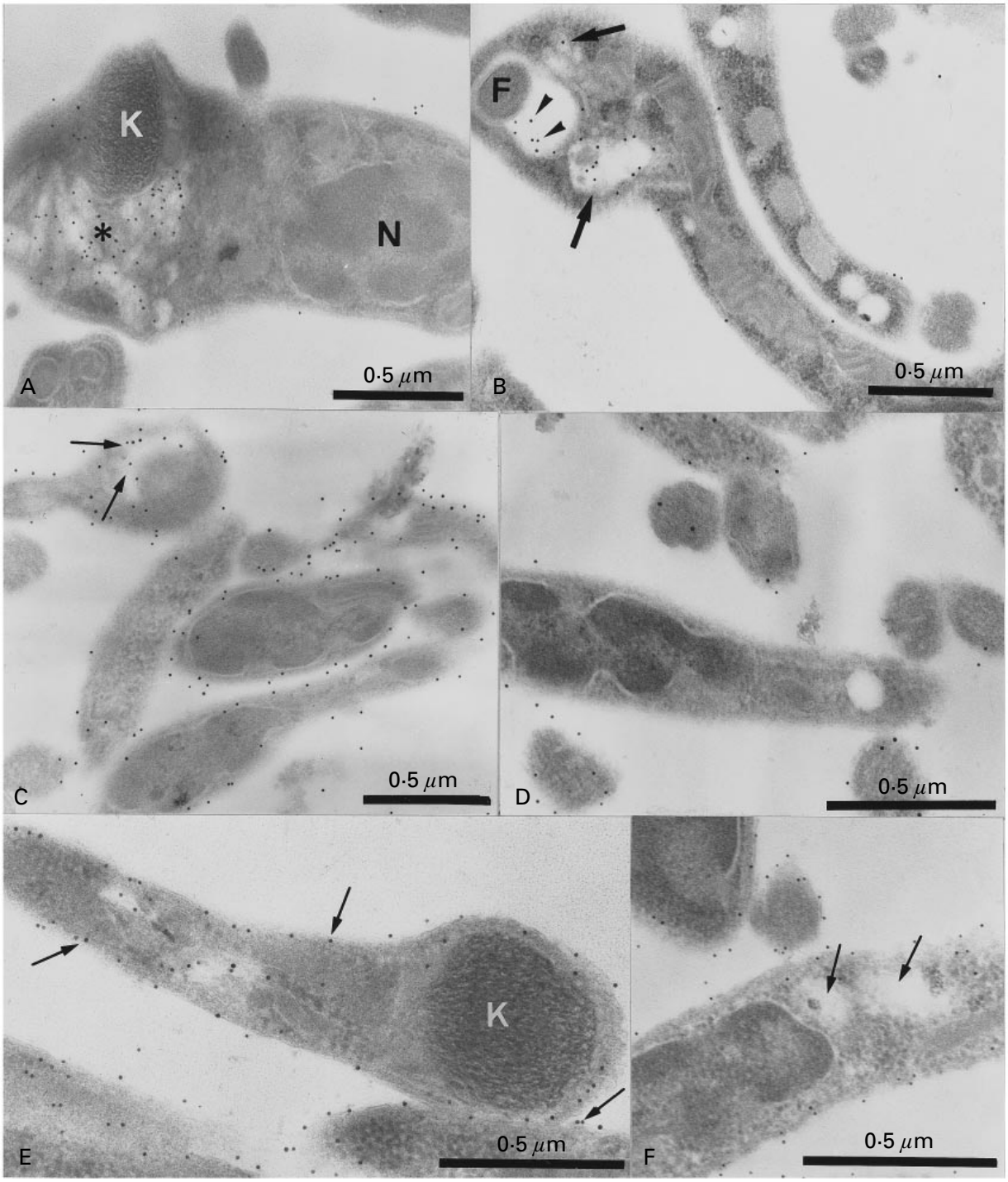


Fig. 1. Immunocytochemical labelling of tissue culture trypomastigotes with a polyclonal antibody against the COOH-terminal portion (R1) of the cysteine proteinase. (A and B) Y strain trypomastigotes. Note the presence of a few gold particles on the surface of Y strain, while a lot of particles are seen inside vesicles distributed throughout the cytoplasm (* in A), on vesicles located close to the flagellar pocket (arrows in B), and inside the pocket (arrowheads in B). (C and D) CL strain trypomastigotes. These parasites show either an intense (C) or a poor (D) labelling of membrane surface. Gold particles are also seen inside the flagellar pocket (arrows in C). (E and F) Clone CL-14 trypomastigotes. Note the intense labelling on the cell surface of CL-14 and the faint labelling of cytoplasmic vesicles (arrows in F), as compared with that observed in Y strain. N, nucleus; K, kinetoplast; F, flagellum.

tightly packed row of fibres perpendicularly oriented in relation to the longitudinal axis of the protozoan.

Labelling of the Y strain parasites with polyclonal antibody against the carboxy-terminal portion of the cysteine proteinase (R1) agrees with that previously described by Souto-Padrón *et al*. (1990): tissue culture trypomastigotes presented a faint labelling of the cellular and flagellar membranes by anti-R1 antibodies, but the intracellular vacuoles located close to the flagellar pocket region and the pocket

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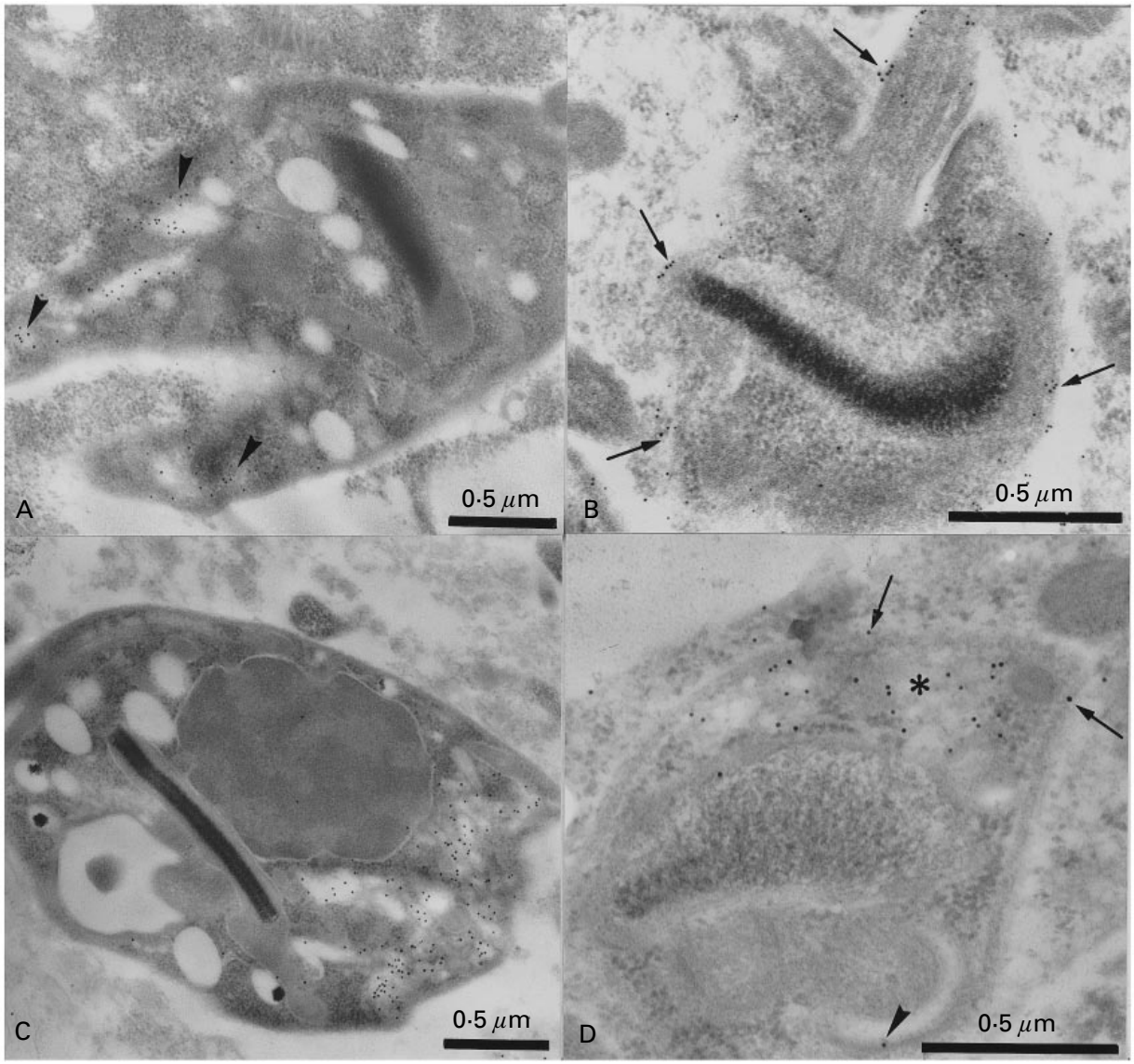


Fig. 2. Immunocytochemical staining of intracellular amastigote forms with polyclonal antibodies against R1 and R2 portions of the cysteine proteinase. (A and B) labelling with anti-R1 antibody. Note the presence of few or no particles on the surface of Y strain (A), whereas CL-14 present a lot of gold particles on the cell surface (arrows in B). Labelling of cytoplasmic vesicles is evident in Y strain (arrowheads in A). (C and D) labelling with anti-R2 antibody. Y strain (C) presents the same pattern of labelling described for anti-R1 antibody while clone Cl-14 (D) shows a lot of particles inside cytoplasmic vesicles (* in D) and a few particles on the cell surface (arrows in D) and flagellar pocket membranes (arrowheads in D).

interior were intensely labelled (Fig. 1A, B). Unexpectedly, tissue culture trypomastigotes of the CL strain presented a different labelling pattern: gold particles were preferentially located on the cell surface and flagellar membranes (Fig. 1C), while a few particles were observed inside the flagellar pocket, associated with the flagellar pocket membrane or inside cytoplasmic vesicles. Moreover, the expression of cruzipain on CL strain trypomastigotes, as detected by anti-R1 antibody, was not uniform: some parasites were completely devoid of gold particles (Fig. 1D). Anti-R1 labelling was preferentially located on the cell surface of clone CL-14 trypomastigotes (Fig. 1E, F), similar to the pattern found in parental CL strain, but the population of CL-14 parasites was very homogeneous, and no parasites devoid of anti-R1 reactivity were observed. The intracellular compartments of CL-14 parasites contained few or no gold particles (Fig. 1F). An antibody directed to the catalytic portion of cysteine proteinase (R2) was also used to investigate the expression of cruzipain on Y and CL strains and clone CL-14, yielding similar results (results not shown).

Labelling of amastigote forms with anti-R1 showed results similar to that observed in trypomastigotes. Intracellular amastigote forms from the Y strain presented numerous gold particles inside cytoplasmic vesicles and few particles on the cell surface (Fig. 2A). This labelling pattern differs from

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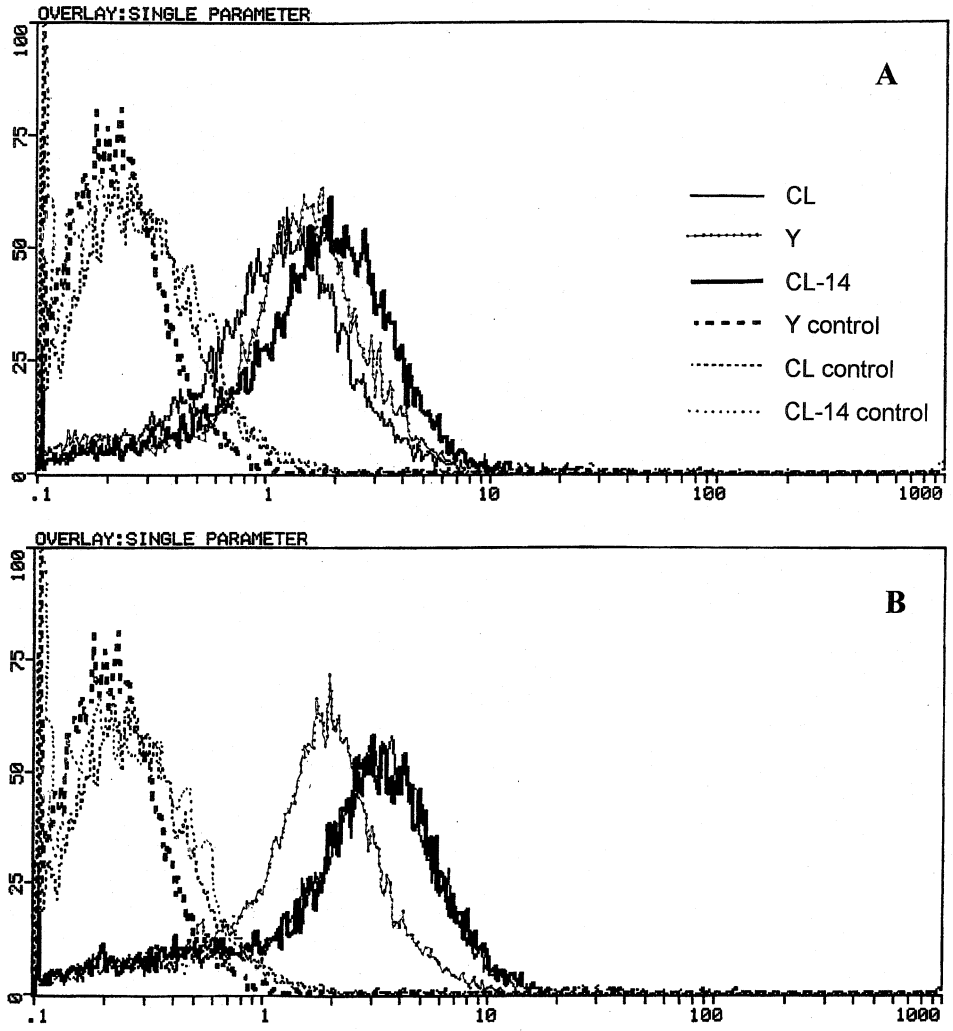


Fig. 3. Flow cytometric analysis of cruzipain expression. Trypomastigote forms from clone CL-14, Y and CL strains were labelled with antibodies directed against R1 (A) or R2 (B) domains of cruzipain. Labelling controls were prepared by substituting PBS–BSA for the incubation with primary antibodies after blocking immunoglobulin receptors.

that described for Y amastigote–trypomastigote transitional forms, in which cruzipain was mainly located on cell surface (Souto-Padrón *et al*. 1990). On the other hand, labelling of intracellular amastigotes from clone CL-14 was mainly located as clusters on the cell surface and flagellar membranes (Fig. 2B). Labelling of intracellular amastigotes from Y strain with anti-R2 was similar to that obtained with anti-R1 (Fig. 2C). However, intracellular amastigotes from the clone CL-14 presented a pattern of anti-R2 labelling different from that observed with R1 antibody: gold particles were mainly located inside cytoplasmic vesicles, similar to Y strain intracellular amastigotes (Fig. 2D).

*Flow cytometry analysis of cruzipain expression*

Reactivity of trypomastigotes from clone CL-14, Y and CL strains to antibodies directed to cysteine proteinase carboxy-terminal (R1) or catalytic (R2) portions was also evaluated by flow cytometry. The results presented on Fig. 3A show that clone CL-14 was strongly labelled by anti-R1, while Y and CL strains present a less intense labelling with this antibody. Clone CL-14 and CL strain displayed the same anti-R2 reactivity, while the reactivity of Y strain to this antibody was less intense (Fig. 3B).

*Cysteine proteinase activity*

To investigate whether the cysteine proteinase presented by trypomastigotes of clone CL-14 was functional and similar to that presented by infective strains, proteinase activity of parasites from clone CL-14, Y and CL strains were assayed on SDS gels using gelatin as substrate. Fig. 4A shows that both infective strains and clone CL-14 presented proteinase activity. The cruzipain expressed by CL-14 presented a double-band pattern, similar to virulent Y and CL strains and to that previously described for trypomastigotes from other strains (Campetella *et al*. 1990). However, these bands presented higher

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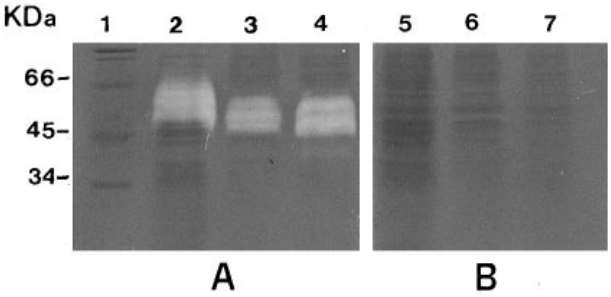


Fig. 4. Proteinase activity depicted in a gelatin gel. Gel is representative of 4 similar experiments. Each lane was loaded with freshly prepared trypomastigote extracts ($10^7$ parasites/lane) from clone CL-14 (2 and 5), CL (3 and 6) and Y (4 and 7) strains, in the absence (A) or presence (B) of 20 $\mu$M E-64. Positions of molecular markers (lane 1) are indicated.

molecular weights than that of infective strains, suggesting the expression of a different enzyme isoform by CL-14. Fig. 4B confirms that cysteine proteinase is the enzyme responsible for this proteinase activity, as it can be blocked by E-64, a specific cysteine proteinase inhibitor.

## DISCUSSION

Expression of cruzipain by CL-14, a non-infective and non-pathogenic clone of *T. cruzi*, was the focus of this study, the underlying premise being that cruzipain is important for parasite infectivity and induction of pathology. Our data show that trypomastigotes of this clone express high levels of a functional cruzipain located on the cell surface, a distribution also found in the virulent parental CL strain. Nevertheless, the gel banding pattern of CL-14 cruzipain differs from that displayed by infective and pathogenic strains, suggesting the expression of a different isoform.

The relation between cruzipain subcellular localization and its functional activity is still not clear. Previous studies have demonstrated that cruzipain is required for cellular invasion, intracellular multiplication and parasite differentiation (Franke-de-Cazzulo *et al*. 1994; Harth *et al*. 1993; McGrath *et al*. 1995, Meirelles *et al*. 1992), but whether the enzyme involved in these processes is located in endosomal–lysosomal compartments remains unknown. In this study, we failed to find a correlation between subcellular cruzipain distribution and infective capacity, since CL-14 and CL present similar subcellular expression patterns. Nevertheless, we found that 2 infective strains (CL and Y) present different cruzipain expression patterns. It remains to be established whether surface cruzipain, which is preferentially expressed by CL-14 and CL, is an isotype involved in different functions, as previously proposed (Scharfstein, 1993; Tomas, Miles & Kelly, 1997).

Clone MHOM/BR/78/Sylvio-X10.6 presents a distribution of cruzipain on trypomastigote forms similar to Y strain, but surface labelling of intracellular amastigotes (Tomas *et al*. 1997). Nevertheless, intracellular amastigotes from the Y strain present cruzipain expression concentrated in internal compartments, adding complexity to the present knowledge of stage-specific patterns of cruzipain expression (Campetella *et al*. 1990; Eakin *et al*. 1992). Intriguingly, we found that intracellular amastigotes from clone CL-14 express R1 domain of cruzipain (carboxy-terminal) on the cellular membrane, while catalytic R2 domain is expressed in internal compartments. The biological existence of separate carboxy-terminal and catalytic domains, if confirmed, would bring a new perspective to the study of cruzipain function.

The hypothesis of cruzipain involvement in the pathogenesis of Chagas' disease has been raised on the basis of the strong immunological response presented by chronic chagasic patients against cruzipain epitopes (Arnholdt *et al*. 1993; Gazzinelli *et al*. 1990; Martinez *et al*. 1991, 1993). In fact, the homology between human lysosomal cathepsin and cruzipain is noteworthy (reviewed by Cazzulo, Stoka & Turk, 1997), and depots of cruzipain were recently found among heart infiltrating mononuclear cells in chronic chagasic patients (Morrot *et al*. 1997), suggesting that cross-reactive responses to these molecules can have a major role in the induction of pathology. T cell lines reactive to both human lysosomal cathepsin and cruzipain were obtained from chagasic individuals (de Araújo *et al*. 1996) reinforcing this hypothesis. Nevertheless, our data argue against a role for cruzipain as a target of pathogenic immunological responses, since expression of high levels of functional cruzipain does not endow clone CL-14 with pathogenicity. Two other hypotheses could explain this paradox. The primary defect of clone CL-14 could lie in its inability to infect cells *in vivo*, leading to rapid clearance of cruzipain, thereby sparing the host from its pathogenic effect. Although we can not exclude this possibility, it is worth mentioning that infection is not necessary to induction of heart inflammatory infiltration by non-adjuvated *T. cruzi* antigens (Ruiz *et al*. 1985), and that the number of CL-14 trypomastigotes inoculated in histopathological studies is large enough to induce efficient protective immune responses against *T. cruzi* (Lima *et al*. 1991). Since neither histopathological lesions nor inflammatory tissue infiltrates could be observed any time after CL-14 inoculation (Lima *et al*. 1995), it should be assumed that long-term persistence of cruzipain is required to elicit immunopathological responses, rather than immediate enzymatic ability to cause tissue damage or inflammation (Svensjo *et al*. 1997). Another plausible hypothesis is that cruzipain expressed by CL-14 presents a different

activity or immunogenicity, making it unable to induce tissue damage, inflammation or abnormal immunological responses.

In summary, results demonstrated that trypomastigotes from *T. cruzi* clone CL-14 present cruzipain expression, subcellular distribution and functional activity comparable to that of infective and pathogenic parental strain, ruling out these possibilities as an explanation for its avirulence. These results cast doubt on the role of cruzipain in the induction of immunopathological responses, since high levels of functional cruzipain are expressed by an apathogenic *T. cruzi* clone. Nevertheless, the banding pattern of CL-14 cruzipain differed from both infective strains, suggesting the expression of a different cruzipain isoform by this clone.

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