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FUNCTIONAL AND ANTIGENIC PROPERTIES OF THE MAJOR CYSTEINE PROTEINASE (GP57/51) OF *TRYPANOSOMA CRUZI*

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In a recent study (Murta et al., 1990), we demonstrated that GP57/51 antigen (Scharfstein et al., 1986; Gazzinelli et al., 1990) is a functionally active cysteine proteinase of *Trypanosoma cruzi*. Two independent research lines, respectively focusing on the structural and functional characterization of *T. cruzi* antigens and proteinases (Cazzulo et al., 1989), have drawn attention to the multi-faceted roles which this highly antigenic proteinase may possibly play in chagasic pathophysiology. In the first part of the present article, we will review information concerning the structure, function and biology of this proteinase. The perspectives of designing new trypanocidal drugs exploring the proteinase's sensitivity to thiol protease specific inhibitors will be briefly discussed, and the potential of this approach will be illustrated by *in vitro* infection assays carried out in the presence of peptidyl diazomethane inhibitors.

In the last part of this article, we will critically discuss the performance of diagnostic kits prepared with purified GP57/51 (WHO-TDR sponsored Multicentre Study). The stability properties of the kit were investigated; we found that there was a significant decrease in the sensitivity of GP57/51 ELISA kits stored for more than 3 months at room temperature. Tests performed with freshly-coated plates confirmed that the antigen was perfectly capable of detecting CH samples (6 out of 29) missed at the time of the WHO-TDR study. A simple method to reactivate the decaying kit was developed, indicating that reversible molecular interactions may take place in Ag-containing plastic wells. Distribution to blood centers should be initiated as soon as best storage conditions are defined.

Characterization of GP57/51 antigen as the major cysteine proteinase – In a recent study (Murta et al., 1990) identified GP57/51 antigen as the Y strain cysteine proteinase. This was shown by structural (N-terminal protein sequence, 30 residues) and functional analysis of the purified glycoprotein. The data indicated that the proteinase function and GP57/51 epitope markers were expressed by the same molecular entities. The N-teminal sequence of the antigen (66.7% homologous to human cathepsin L, a lysosomal proteinase) contained the reactive site cysteine residue, and was virtually identical (difference in one single amino acid residue) to that reported for the 60 kDa Tulahuen proteinase characterized by Cazzulo et al. (1989). Moreover, our sequence was identical to the partial DNA sequence reported by Eakins et al. (1990), whose studies clearly assigned this protein to the papain superfamily. The proposition that GP57/51 is the Y strain homologue of the Tulahuen proteinase was corroborated by data showing that these enzymes were both sensitive to thiol protease inhibitors, and that their kinetic parameters are similar (Murta et al., in press; Cazzulo et al., 1990; Lima et al., in preparation). Differences of pH optima may stem from the use of different protein substrates; in our hands, the Y strain GP57/51 protease readily digests IgG and gelatin at neutral/slighly acidic pH.

The 57/51 kDa Y strain proteinase (and most likely the 60 kDa Tulahuen enzyme) are

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probably related to a partially characterized protease described by Rangel et al. (1978) several years ago. In fact, it probably corresponds to the major cysteine proteinase bands which Greig and Ashall defined in *T. cruzi* crude extracts run on SDS-PAGE gels co-polymerized with gelatin substrate; its migration as a 38-40 kDa band (usually a doublet) under these particular conditions of electrophoresis (non-denaturing, non-reducing) is caused by folding of the proteinase (Murta et al., 1990).

One should expect a marked degree of conservation of this proteinase in the *T. cruzi* species, if the immunochemical data relative to GP57/51 (Scharfstein et al., 1985; 1986) proves extensive to the enzyme. By the former criteria, antigenically related products were detected in all developmental forms, including bloodstream trypomastigotes (Scharfstein et al., 1986) and amastigotes (unpublished observations). The structural basis for Mr and pI differences of purified GP57/51 is not well defined, as yet. These components may represent different processing forms, as recently suggested by pulse-chase experiments performed in a defined system of metacyclogenesis *in vitro* (Bonaldo et al., submitted to publication). The multiple isoelectric forms (4.5-4.8 pI range) identified on Dm28c clones may reflect the expression of isoforms and/or differential postranslational modifications, perhaps determined by the N-linked (Cazzulo et al., 1990) or O-linked oligosaccharide chains (Mendonça-Previato et al., 1983); it is noteworthy that the % of glycoconjugates in this glycoprotein may be considerably higher than the 10% proposed by Cazzulo et al. (1990b), if we assume that O-oligosaccharide chains identified earlier in GP25 fragment(s) (40%, as determined by Mendonça-Previato et al., 1983) are also present in GP57/51. Interestingly, Bonaldo and co-workers have recently showed that the expression of GP57/51 antigen/proteinase is developmentally controlled during metacyclogenesis (submitted).

Ultrastructural localization of GP57/51 antigen – The sub-cellular localization of GP57/51-related products was investigated with monoclonal antibodies, which localized them in vesicles of the endosomal/lysosomal system and in the flagellar pocket, in trypomastigotes as well as in epimastigotes (Murta et al., 1990), in agreement with data obtained after biochemical fractionation (Bontempi et al., 1989). The presence of immunoreactive products in the flagelar pocket suggests that the antigen (perhaps the active proteinase) can be targeted for secretion. It is noteworthy that surface immunoreactivity is very discrete when monoclonal, rather than polyclonal antibodies (Souto-Padron et al., 1990; Scharfstein et al., 1983) were used in immunoelectronmicroscopy studies. Previous analysis of surface radioiodinated proteins from trypomastigotes had already indicated that GP57/51 was not accessible to antibodies, in contrast to observations made in epimastigotes (Scharfstein et al., 1986). Admittedly, it is unlikely that trypomastigotes would carry significant quantities of this proteinase at the surface, inasmuch as they would probably render the parasites sensitive to antibody-dependent effector responses; these restrictions would obviously not operate for the insect stages, where the antigen can be indeed detected.

Possible pathological implications – It will be important to determine if the cysteine proteinase is released to extracellular fluids, and if so, under which conditions. In addition to active secretion, this highly antigenic proteinase may leak from damaged parasites, thereby contributing to inflammation and tissue damage. The studies on the cysteine proteinases from *Entamoeba histolytica* sets an interesting precedent; one of these enzymes can activate Complement C3 (Reed et al., 1989), and in addition it can promote the degradation of connective tissue extracellular matrix, type I collagen, laminin and fibronectin (Keene et al., 1986). In Chagas' disease, tissue pathology could be further amplified by mononuclear cell infiltrates recruited to sites of infection by antigen specific T cells. We now know that in addition to antibody responses (Scharfstein et al., 1985), GP57/51 elicits vigorous proliferative responses in the large majority of chronic chagasic patients (Gazzinelli et al., 1990; Arnholdt et al., 1990). Hence, these independent biological activities may contribute to chronic inflammatory lesions. The functional (pattern of secreted lymphokines) and phenotypic properties of the responding T cells lines/clones are presently under investigation.

Another fundamental problem to be tackled is the structural characterization of the peptide epitopes recognized by GP57/51 specific T cells. As a member of the papain superfamily, GP57/51 is expected to show considerable structural homology with mammalian cathepsin

L/B and H (ref. to Eakins et al., 1990) and to a lesser degree with Calpains I/II, the calcium activated group of cytoplasmic proteinases. To investigate if T cell epitopes of GP57/51 are contained within the highly conserved regions of the papain superfamily, we carried cross-immunization studies with papain, the protypic cysteine proteinase. Our data (Arnholdt et al., 1990) showed that spleen cells obtained from mice immunized with GP57/51 develop low, albeit significant T cell proliferative responses against papain, *in vitro;* conversely, mice immunized with papain were cross stimulated with purified GP57/51. If confirmed, these preliminary data suggest that the sequences shared by these two members of the cysteine protease family encompass common T cell epitope(s). We then set out to investigate if chronic chagasic patients and normal individuals recognize papain. The results from this limited survey (Arnholdt et al., 1990) indicate that the patient's T cells respond vigorously against purified GP57/51, and also to papain. Cells from normal donors (survey limited to a few cases thus far) did not respond to papain, or as expected, to GP57/51. T cell cloning is being presently sought to verify if these responding lymphocytes contain subpopulations with overlapping specificities. Access to these clones may be instrumental to the identification of T cell recognition units within members of the cysteine proteinase family, perhaps including host tissue molecules.

Cysteine proteinases as targets for novel trypanocidal drugs — In the past few years, a growing list of parasites have been shown to express thiol proteases (reviewed by North et al., 1990a), in keeping with the notion that members of this protein family are widely distributed throughout living systems, being found also in plants, animals, virus and bacteria. Among the various specific inactivators of cysteine proteinases, the peptidyldiazomethanes [PDM] (Leary et al., 1977) have drawn attention for their discriminatory effects for cathepsins B, cathepsin L and calpains (Crawford et al., 1988). The PDM alkylate the reactive site cysteine residue, the selectivity of each compound being determined by the peptide portion of the inhibitor.. To investigate the effects of PDM inhibitors as potential trypanocidal drugs (refer to Meirelles et al., 1990), the following compounds were synthesized: Bz-phe-gly-CHN2, Z-phe-phe-CHN2, and Z-[S-Bz].cyz-phe-CHN2. Rate constants for inactivation of the purified enzyme were determined by use of continuous assays in the presence of inhibitor and substrate as described by Tian & Tsou (1982), using Z-phe-arg-NMec, as described by Murta et al. (1990). In decreasing order, the most effective inhibitors of the enzyme were Z-[S-Bz]cys-phe-CHN2 (Ki 0.58 uM), Z-phe-phe-CNH2 (Ki 1.30 uM) and BZ-Phe-gly-CHN2 (Ki 6.4 uM). Various tissue culture systems were used to assess the potential value of these PDM's as tryponacidal drugs: primary cultures of heart muscle cells (HMC), Vero (fibroblastoid) and LLCMK2 (epithelial line) were used alternatively as host cells, whereas bloodstream trypomastigotes (Y strain or Dm28c) were used to promote infection. Screening was initially carried out with HMC, 24 h after addition of the parasite inoculum to the cell culture. The culture were washed, and supplemented with medium containing each one of the PDM, at a fixed concentration (10 uM). The PDM-supplemented medium was changed every 24 h, and the cells were fixed and stained with Giemsa at every time interval (24 h). Part of our results are shown in Table I; the first indications of parasitological activity were noted only 72 h after the onset of cultures: Z-[S-Bz].cyz-phe-CHN2 (59% inhibition relative to cultures supplemented with PDM solvent) and Z-phe-phe-CHN2 (28% inhibition) markedly inhibit the development of intracellular infection. Interestingly, the parasites were swollen, and the differentiation process was inhibited in relation to controls (not shown). The degenerating intracellular forms disappear with time; the cells were completely free of parasites after 144 h of culture, in the presence of Z-[S-Bz]. cys-phe-CHN2. By then, parasite burden was noticeable, but markedly reduced, with the second most active inhibitor, Z-phe-phe-CHN2. Consistent with these observations, we did not find any trypomastigotes in fluids from cultures treated with Z-[S-Bz].cyz-phe-CHN2; they could be found in very low numbers, however, in cultures treated with Z-phe-phe-CHN2. There was no obvious indication of toxicity in any of the host cell systems investigated. In a second protocol, the most active inhibitors were tested in an interaction system, to determine if these compounds could interfere with the invasive capacity of trypomastigotes. Indeed, these experiments (Table II) indicated that pre-treatment with the drug impaired the parasite's ability to infect HMC. Furthermore, pre-treatment of parasites of HMC cells sepa-

TABLE I

Effect of peptidyl diazomethane inhibitors on heart muscle cells infected 24 h earlier with *Trypanosoma cruzi*[a]

Peptidyl diazomethane (PDM)	Conc.	Infection index (72 h culture)	Trypomastigotes[b] intracellular/supernatant	
	(uM)		144 h	144 h
Z-(S-Bz)cys-phe-CHN2	10	0.41 (no tryps)	not found	not found
Z-phe-phe-CHN2	10	0.72 (few tryps)	few tryps	not found
DMSO-solvent	/	1.0 (plenty tryps)	plenty	plenty
Medium only	/	1.0 (plenty tryps)	plenty	plenty

a: Y strain, bloodstream forms.
b: Tryps were removed from cultures 24 h after onset of incubation, and HMC were supplemented with PDM-rich medium thereafter (fresh supplements added each 24 h).

TABLE II

Effect of Z-(S-Bz)cys-phe-CHN2 on the interaction of heart muscle cells with *Trypanosoma cruzi*

	% Infected cells[a]	
Type of cell(s) exposed to drug	2 h treatment	20 h treatment
none (control with medium)	6	17
Trypanosoma cruzi only	4	5
heart muscle cells only	4	12.5
T. cruzi and heart muscle together	3	3

a: tryps were treated with 10 uM of Z-(S-Bz)cys-phe-CHN2 for 30 min, washed off inhibitor by centrifugation, and incubated for 2 or 20 h with HMC, the cells being fixed and the % counted at the end of the incubation period (Giemsa stain). Experiments were run in triplicates.

TABLE III

IC-50 values (nM)
Z-(S-Bz)cys-phe-CHN2

Type of host cell	IC-50
LLCK2	150 nM
VERO	480 nM

Bloodstream tryps. (Y str) and host cells were incubated with decreasing conc. of the pep-diazomethane in complete medium (10-0.015 uM range). After 24 h of incubation, the parasites were removed, and the cultures were supplemented with medium containing the drug (or DMSO-diluent) at the same concentration. The % of cells showing pseudocysts was scored after 4 to 5 days of culture.

rately with these drugs suggested that the inhibition was due to the action of the inhibitors on the parasites, rather than on HMC.

We next set out to determine dose response curves for Z-[S-Bz].cys-phe-CHN2, which this time was added to the cultures as off the start of the experiment. The cultures were washed 24 h later, and the PDM containing medium was re-introduced to the cultures, which were not manipulated thereafter. The results shown in Table III represent the concentration of drug necessary to inhibit infectivity to 50% of controls (IC50), that is, 150 to 500 nM depending on the host cell type used (LLCMK2/VERO). There was no evidence of pseudocyst development at concentrations above 1.2 uM of this PDM. Ultrastructural analysis of intracellular and extracellular parasites has shown swelling and displacement of membranes, cytoplasmic loss and the presence of membrane blebs (Meirelles et al., in preparation).

Our data suggest that there is a direct correlation between the activity of PDM's as cysteine proteinase inhibitors and their trypanocidal activity *in vitro*. Additional experiments are required to demonstrate that GP57/51 (alone, or perhaps together with other cysteine peptidases, ref. to Greig & Ashall, 1990) is indeed the ultimate target of the trypanocydal PDM's. There is no question,

however, that Z-[S-Bz]cys-phe-CHN2 and Z-phe-phe-CHN2 can reach parasites within infected cells, and block their differentiation into infective trypomastigotes. Moreover, the inhibitors appear to impair functio(s) required for host cell penetration. Their effectiveness as enzyme inhibitors probably reflect the presence of bulky hydrophobic residues in the P2 position, in addition to P1, this evidence being thus consistent with the proposition that GP57/51 is indeed closely related to mammalian cathepsin L. One may expect that the study of this protein's structural/functional relationships may lead to the design of increasingly selective trypanocidal drugs.

Diagnostic kits prepared with purified GP57/51: perspectives for application — Here we discuss the performance of diagnostic kits prepared with purified GP57/51, in the WHO-TDR Multicentre Study. As reported in this meeting, our data showed an overall agreement of 0.88, and Kappa index of 0.77. The recognition that the assay performed beyond expectations have prompted us to re-evaluate the reaction conditions with the original plates (8,000 microwells were sensitized early in December 1989, dried, and distributed to 2 different hemocenters/S.P. and NYC; the unsealed plates were stored at room temperature, thus were exposed to normal atmosphere as off the date of plating), and compare its performance with freshly sensitized microplates (same batch of purified GP57/51). Retrospective analysis of P/N values obtained during this time interval (4 months) suggested that the kit had lost its sensitivity by the time the GOWH serum panel was tested (data provided by Dr A. Khoury, Hemosoro/SP). We then sought to determine if the decreased reactivity was due to reversible or irreversible processes; indeed the original plates recovered at least part of its lost reactivity (as defined by a monoclonal antibody) upon brief exposure to heat (4 min, microwave oven, data not shown). We then considered the possibility that the 6 serum samples (out of 29 CH-samples) missed in our earlier participation in the WHO-TDR Multicentre Study (GOWH/14, 15, 26, 41, 47, 48) actually contained specific antibodies to GP57/51. Five of these samples (GOWH/14, 15, 26, 47, 48) were accessible (kindly supplied by Dr A. Luquetti), and could be tested again. The results shown in Table IV demonstrate that these sera indeed react with GP57/51 antigen when tested with freshly coated plates; tests performed with reactivated kits yielded weaker signals, but significantly above cut-off values. Hence, the detection problems observed with these particular GOWH samples were the consequence of inappropriate storage of the kit, rather than caused by true non-responsiveness to GP57/51. Thus far, the only well established cases of confirmed negative serology were found within a sub-group of patients submitted to chemotherapy, and presumably cured (Gazzinelli et al., unpublished data).

FIG. 1A

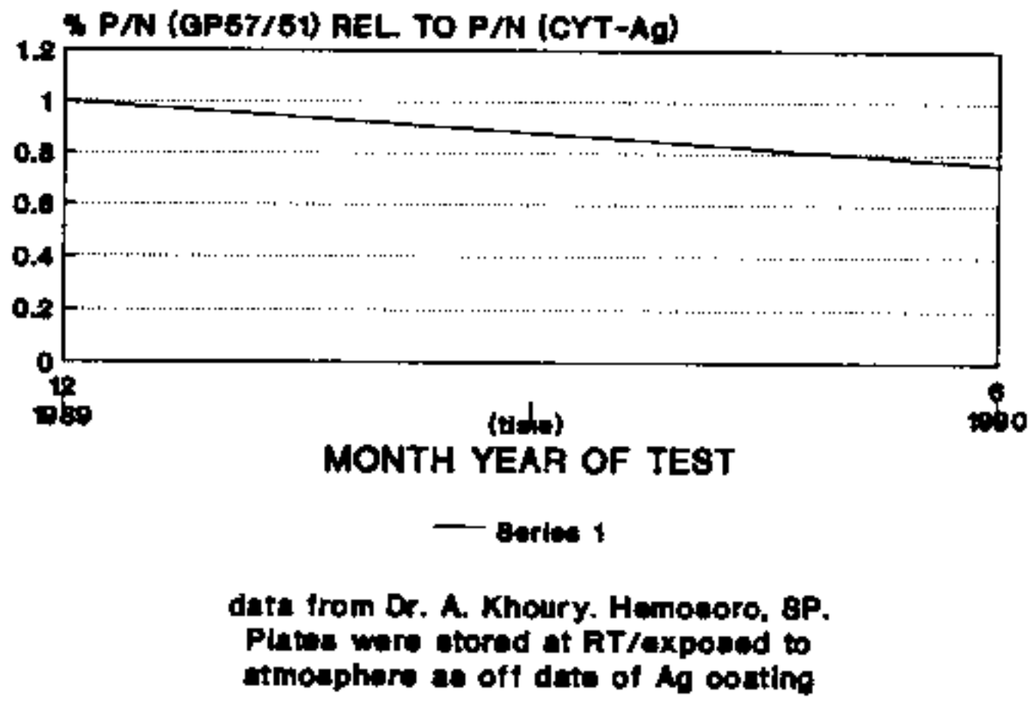
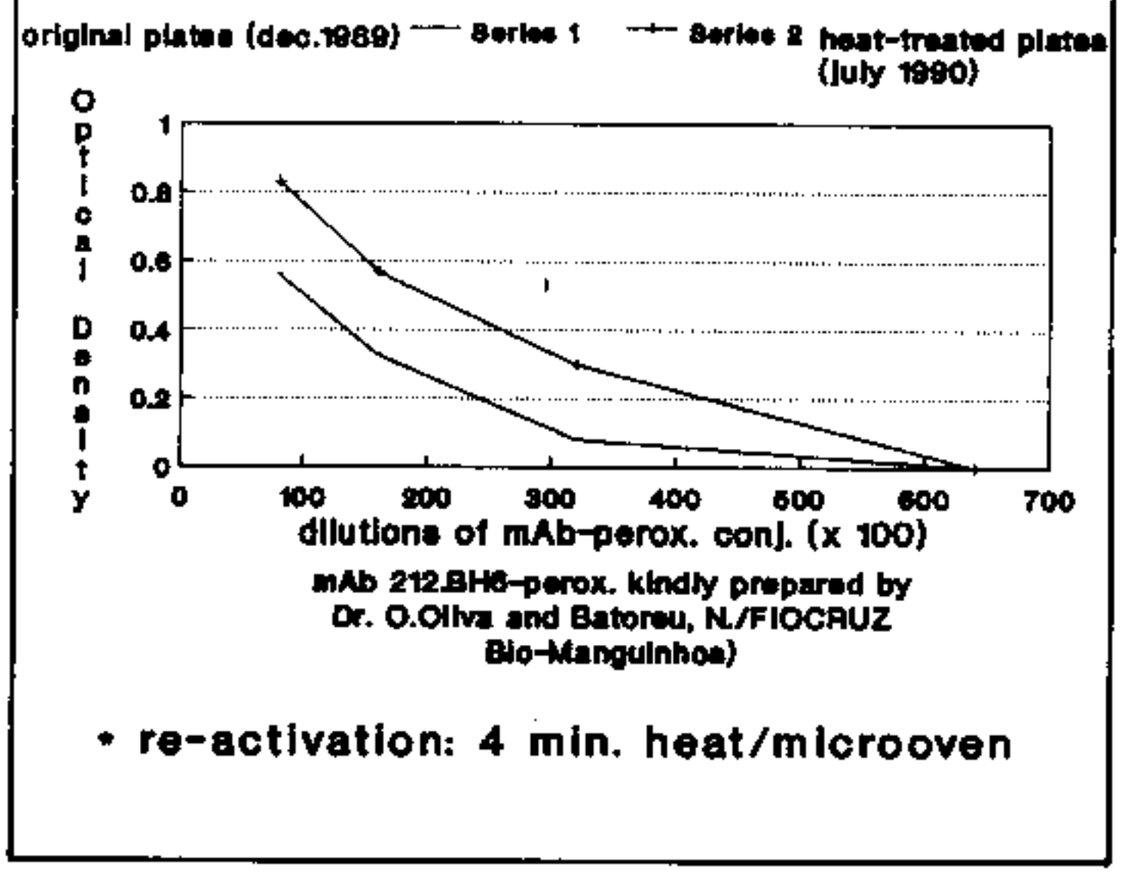


FIG. 1B



The nature of the slowly developing molecular interactions occuring at the solid phase is under investigation; preliminary data suggest that they may differentially affect the expression of GP57/51's epitopes. Interestingly North et

al. (1990b) have recently reported differential instability of HIV1/HIV2 antigens in ELISA kits prepared with HIV1 or HIV1 + HIV2 antigens. Large scale distribution of the kit should be possible as soon as optimal storage conditions are defined.

TABLE IV

Diagnostic performance of GP57/51 kit prepared with freshly coated microplates

Serum origin	WHO-TDR Study	Fresh-plates	Conclusion
Ref. Classif.	Pos./Neg.	O.D. (492)/SD	Pos./Neg.
Ref. Chag.[a]	Ref. +	0.268(0.009)	Ref. +
Ref. NR[b]	Ref. –	0.067(0.004)	Ref. –
GOWH 14 (CH-H)	Neg.	0.423(0.053)	Pos.
GOWH 15 (CH-L)	Neg.	0.409(0.08)	Pos.
GOWH 26 (CH-L)	Neg.	0.283(0.01)	Pos.
GOWH 41 (CH-H)	Neg.	(not available)	nd
GOWH 47 (CH-L)	Neg.	0.882(0.034)	Pos.
GOWH 48 (CH-L)	Neg.	1.116(0.01)	Pos.
GOWH 37 (NR)[c]	Neg.	0.064(0.01)	Neg.

a: Ref. CH sera 1:3200.
b: Ref. NR sera and GOWH tested at 1:100 final.
c: GOWH 37 (NR) used as internal control of WHO panel. anti-Hu IgG. perox. conj. used at 1:3000.

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