Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *85* (4): 519-522, oct./dec. 1990

USE OF TWO RECOMBINANT PROTEINS OF *TRYPANOSOMA CRUZI* IN THE SEROLOGICAL DIAGNOSIS OF CHAGAS' DISEASE

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INTRODUCTION

Presently, the serological diagnosis of Chagas' disease relies upon the widely used indirect immunofluorescence (IIF) techniques, passive hemagglutination tests and ELISA. The classical complement fixation reaction, developed by Guerreiro and Machado in 1913, is being less used due to difficulties in standardization and lack of a reliable antigen preparation.

Antigens commonly used in conventional serological techniques are whole epimastigote forms or a complex mixture of components extracted from the non-infective stage of the parasite. The specific *Trypanosoma cruzi* strain employed and the different techniques used for antigen extraction introduce an undesirable variability in the reagent preparation. Finally, these reagents may give rise to false-positive reactions mainly due to cross reactivity with antibodies developed against other disease agents.

To overcome these problems one of the obvious alternatives is to use recombinant polypeptides containing antigenic *T. cruzi* specific epitopes which, necessarily, must elicit an immune response in the majority of chagasic patients. Furthermore, these antigens should be common to all known *T. cruzi* strains and should not cross react with sera from patients carrying other diseases. Finally, a good recombinant antigen should be easy to obtain through reproducible methodology and be used, preferably, in conjunction with normally employed laboratory techniques.

In the present communication two recombinant polypeptides from *T. cruzi* are described which, potentially, may be employed in the serological diagnosis of Chagas' disease.

This work was supported by grants of the Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico e Financiadora de Estudos e Projetos.

RESULTS

Description of the recombinant clones – Two recombinant clones expressing *T. cruzi* antigenic determinants were originally isolated from a genomic library constructed in the vector lambda gt11 (Young & Davis, 1983). Screening of the library was performed with a rabbit hyperimmune serum to tissue culture trypomastigotes (Y strain) attenuated with 8-methoxypsoralen (Andrews et al., 1985). In order to increase the amount of antibodies specifically directed to trypomastigotes, the antiserum was previously absorbed with epimastigote forms.

Sequencing of both recombinants, named B-12 and B-13, indicated a pattern of tandemly repeated units of 20 and 12 aminoacids, respectively. The characterization of both clones, as to their genomic arrangement in different strains and clones of *T. cruzi*, as well as the corresponding mRNA transcripts and sequence data will be presented elsewhere (Gruber & Zingales, manuscript in preparation). Comparison of the clones sequences with data from previously described *T. cruzi* clones, indicated that clone B-13 is homologous to clone 2 isolated by Ibañez et al. (1988) and recombinant TCR 39 described by Hoft et al. (1989), whereas clone B-12 is a non-described recombinant.

Purification of the recombinant polypeptides – In order to obtain high amounts of the fusion polypeptides, the inserts were subcloned in the plasmid pMS gt11 (Scherf et al., 1990) in phase with the lac Z gene. This vector possesses, as an extra-advantage, a sequence coding for the cleavage site of the blood coagulation factor Xa, introduced between the 3' end of the lac Z gene and the 5' end of the cloned insert. This feature allows

the release of the target polypeptide from the carrier molecule beta-galactosidase by specific treatment with factor Xa (cf. Scherf et al., 1990).

Recombinant proteins, corresponding to the B-12 and B-13 clones, were expressed in the host *Escherichia coli* K12 DH5 alfa in LB medium without addition of IPTG (isopropyl beta-D-thiogalactopyranoside), since beta-galactosidase is constitutively expressed in this system. Purification of the fused polypeptides from the bacteria was achieved after lysozyme treatment followed by sonic vibration and affinity chromatography on pAPTG-agarose columns (p-aminophenyl-beta-D-thiogalactopyranoside). Polypeptides were eluted with 0.1 M sodium borate buffer, pH 10.0 (Steers et al., 1971). Since the foreign antigens were cloned at the extreme end of the C-terminus of beta-galactosidase the elution of the recombinant polypeptides could be easily monitored by determination of the enzymatic activity (cf. Ruther & Müller-Hill, 1983; Scherf et al., 1990). Under the conditions employed the yield was approximately 50 mg of pure recombinant protein/liter of culture medium. The results of several independent purifications indicated that the procedure is very reproducible.

Radioimmunoassay for serological diagnosis The evaluation of the recombinant B-12 and B-13 proteins as reagents for the diagnosis of Chagas' disease has been made initially by radioimmunoassay (RIA). Calibration curves with standard normal and chagasic human sera indicated that amounts of 20-30 ng of either recombinant protein, fixed in wells of flexible 96-well plates (Falcon 3911), were suitable for RIA. It has also been observed that normal and chagasic sera have negligible amounts of antibodies to beta-galactosidase, isolated from non-recombinant pMs gt11. After binding of the fusion polypeptides, the wells were blocked with PBS (10 mM sodium phosphate buffer, pH 7.2) containing 2% BSA (bovine serum albumin). Alternatively, PBS-5% defatted milk was used as blocking solution. The human sera were assayed in a 1:100 dilution and immunecomplexes were quantified after addition of radioiodinated protein A (with either ^{131}I or ^{125}I; 2×10^5 cpm/well). Radioactivity was determined in a gamma counter.

Screening of human sera – An initial survey consisted of 20 normal and 70 chagasic sera, screened by RIA at 1:100 serum dilution. This procedure allowed to discriminate between positive and negative results using either recombinant *T. cruzi* polypeptide. Those samples giving results which did not allow a clear distinction between negativity and positivity were rescreened using 3 serum dilutions (1:25, 1:50 and 1:100). Statistical analysis of the data was performed according to the method adopted by WHO for the Multicentric Study to test recombinant proteins for diagnosis. Kappa indices, which measure the proportion of true agreements (e.g. observed agreements corrected against chance expected agreements) were 0.96 and 0.60, respectively, for B-13 and B-12 recombinant antigens. Since in the present survey it has been observed that some chagasic sera reacted with one of the polypeptides and were negative to the other (regardless whether the antigen was B-13 or B-12), the hypothesis was considered that such differential reactivity could be ascribed to the clinical manifestation of the disease. To test this hypothesis, 36 chagasic sera from patients with well defined clinical diagnosis have been analysed (13 cardiopaths, 13 asymptomatics and 10 having the digestive form of the disease). No preferential reaction with either B-13 or B-12 antigen was observed.

We also investigated whether the differences in the reactivity to the antigens could derive from the *T. cruzi* strain harbored by the patient. Sera of 12 chagasic patients from the endemic region Bambuí (Minas Gerais, Brazil), classified accordingly to the zymodeme of the *T. cruzi* strain that they harbored (Romanha et al., 1979), have been analyzed. The results shown in Table indicate that no preferential response to B-13 or B-12 could be assigned to a particular zymodeme. In those cases where a false-negative reaction with one of the antigens was observed, sera incubation with both recombinant polypeptides allowed the correct diagnosis (cf. Table).

The specificity of the reaction of B-13 and B-12 antigens towards chagasic sera was checked by reacting sera from patients with leishmaniasis, toxoplasmosis, malaria and schistosomiasis with both antigens.

TABLE

Reactivity to B-13 and B-12 recombinant proteins of sera from patients from Bambuí classified accordingly to the zymodeme of the *Trypanosoma cruzi* strain

Serum sample	Zymodeme[a]	Form	IIF[b] titer (−1)	Reactivity to[c] B12	B13	B12 + B13
1	A	cardiac	360	+	−	+
2	A	cardiac	90	+	+	+
3	A	undetermined	45	+	+	+
4	B	cardiac	360	+	+	+
5	B	cardiac	720	+	+	+
6	B	cardiac	360	+	−	+
7	C	undetermined	180	+	+	+
8	C	cardiac/digestive	720	+	+	+
9	C	undetermined	90	−	+	+
10	D	undetermined	45	−	+	+
11	D	cardiac/digestive	180	+	+	+
12	D	cardiac	45	+	+	+
13	Normal	−	20	−	−	−
14	Normal	−	20	−	−	−
15	Normal	−	20	−	−	−

a: Zymodeme was defined after recovery of the infective strain from the patient and analysis according to Romanha et al. (1979).
b: IIF, indirect immunofluorescence with formalyn-fixed tissue culture trypomastigotes (Y strain).
c: Reactivity by RIA with B-12 (30 ng/well); B-13 (20 ng/well); B-12 + B-13 (30 ng + 20 ng/well, respectively).

CONCLUSIONS AND PROSPECTS

Two recombinant proteins expressing *T. cruzi* antigenic determinants were obtained. The potential use of these proteins (named B-12 and B-13) in the serological diagnosis of Chagas' disease was evaluated in a preliminary screen comprising 20 normal sera and 70 human chagasic sera from patients in the chronic stage of the disease. Kappa indices indicated that polypeptide B-13 (Kappa index = 0.96) is a better target than B-12 (Kappa index = 0.60). However, as far as we can tell from the relatively small number of samples examined, the concomitant incubation of sera with both antigens allowed the correct diagnosis. No preferential reaction with either B-13 or B-12 was observed with sera from chronic patients with the undetermined, cardiac or digestive form of the disease. The reactivity of sera from humans in the acute phase of Chagas' disease will be evaluated in the next future. Although preliminary results suggest that the variation in the reactivity toward either recombinant antigen observed with some sera is not due to the *T. cruzi* strain infecting the patient, this analysis will be further pursued using sera from infected animals harboring known. *T. cruzi* isolates. Also, the possible reactivity with serum from *T. rangeli* infections in animals will be analyzed by the same approach. Furthermore, the systematic screening of blood bank samples will permit to evaluate both antigens as to their reactivity toward human sera of patients with other pathologies such as autoimmune diseases or affections with regional epidemiological importance.

Taken as a whole the results shown make B-13 and B-12 recombinant polypeptides potentially good candidates for the diagnosis of Chagas' disease.

As technical comments on the procedure to obtain and purify the recombinant peptides, it should be reminded that the expression system employed (pMS gt11/E. coli DH5 alfa) is very cheap, since it does not employ IPTG for induction and the purification is made in one-step affinity chromatography. The yield of pure proteins is approximately 50 mg/l of original culture medium. The recombinant proteins contain the cleavage site for the blood coagulation factor Xa, allowing the release of the target polypeptide from the carrier beta-galactosidase, if desired. The detection of immunecomplexes can also be carried by ELISA or dot ELISA assays, the choice of the technique being dependent on the facilities found in each laboratory. Aiming at obtaining

cheaper reagents to be used in field trials coupling of the antigens to other supports will be investigated.

Since both recombinant proteins have tandemly repeated aminoacid epitopes, an alternative reagent for diagnosis could be the synthetic peptides modelled to the sequence of the repeat unit.

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