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USE OF *TRYPANOSOMA CRUZI* RECOMBINANT ANTIGENS IN THE IMMUNOLOGICAL DIAGNOSIS OF CHAGAS' DISEASE.

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INTRODUCTION

Chagas' disease is still a major endemic problem in South America where some 20 million individuals display seropositivity for *Trypanosoma cruzi*, causative agent of the disease (Chagas, 1909). The life-cycle of the parasite involves two intermediary hosts (the triatomine insect and mammals) and three well-defined developmental forms, the infective non-replicative trypomastigotes, the replicative amastigotes (mammalian form) and epimastigotes (insect form) (Brener, 1973). The infection occurs when trypomastigotes, released with the excreta of the triatomine, penetrate into the mammalian host through a wound or mucosa. Alternatively, Chagas' disease can be transmitted by transfusion with infected blood. In recent years, the transmission of Chagas' disease by the triatomine invertebrate host has diminished in virtue of improvements in vector control campaigns in some of the endemic countries. However, new cases of this disease still occur due to blood transfusion with infected blood.

Transfusional Chagas' disease frequently occurs as a result of incomplete or deficient diagnosis of the disease. In a few cases, there is a complete lack of blood control and the transfusion is direct (arm-to-arm). In most cases, only one test is used for the screening of the blood, normally agglutination, which results in many false negative responses due to the poor sensibility of the test (Carrasco et al., 1985). However some

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of the blood is screened using at least two different tests. But even in this case, some false results still arise due to the low sensitivity of some methods or antigens, or cross reactivity of *T. cruzi* extracts used in the diagnosis with other diseases such as leishmaniasis, syphilis, toxoplasmosis, etc. (Camargo & Takeda, 1979).

These problems might be solved by purifying parasite specific antigens from parasite extracts. However, these antigens are not generally available and their production costs would be too high, in addition to provoking the risk of infection of people involved in the manipulation of the parasite.

On the other hand, the cloning and expression of *T. cruzi* genes in bacteria might provide antigens from the parasite with the required specificity and at low cost. Accordingly, recent work from several laboratories has resulted in the cloning and expression of *T. cruzi* genes in *E. coli* (Ibanez et al., 1988; Lafaille et al., 1989; Hoft et al., 1989; Levin et al., 1989; Cotrin et al., 1990; Paranhos et al., 1990). The results presented below describe the cloning and characterization of *T. cruzi* specific antigens and their use in the diagnosis of Chagas's disease.

RESULTS

Cloning of *T. Cruzi* Antigens

The successful attempts to clone and express *T. cruzi* genes in bacteria coincided with the description of the lambda gt11 vector (Young & Davis, 1983). The general strategy consisted of the screening of *T. cruzi* expression libraries with either chagasic sera or antisera raised against the parasite. These expression libraries were either cDNA (Hoft et al., 1989; Levin et al., 1989) or genomic (Peterson et al., 1986; Dragon et al., 1987; Ibanez et al., 1988; Lafaille et al., 1989; Cotrim et al., 1990; Zingales et al., 1990), in the latter case taking advantages of the fact that the genes of the parasite are intronless.

In the work carried out in our laboratory, DNA was extracted from *T. cruzi* epimastigotes and cloned in lambda gt11 after shearing to a mean size of 1kb and subsequent addition of EcoRI linkers. The recombinant DNA library in *E. coli* was immunologically screened with a trypomastigote – specific antiserum and the positive clones were further screened with a pool of chagasic sera (Lafaille et al., 1989). Twelve clones positive with the two sera types were then purified to homogeneity.

In order to investigate the specificity of the recognition of these recombinant clones by chagasic sera, we tested the respective fusion proteins by western blot analysis against different human sera. These sera comprised both chagasic sera and sera from patients bearing diseases which cross-react antigenically with Chagas' disease. In this selection, two of the 12 recombinant clones were considered specific for Chagas' disease diagnosis.

Characterization of the Clones

The selected recombinant clones were characterized in terms of their structure and expression (Lafaille et al, 1989; Krieger et al., 1990). One of the antigens is located in the region of the flagellum adjacent to the body of the parasite, while the other is distributed in the cytoplasm of *T. cruzi*. Nucleotide sequencing analysis demonstrated that both antigens are composed of repetitive epitopes: the flagellar antigen is composed of

repetitions of a 68 aminoacid motif, while the repeat unit of the cytoplasmic antigen contains 14 aminoacids. These antigens were then named FRA (flagellar repetitive antigen) and CRA (Cytoplasmic repetitive antigen) (Lafaille et al., 1989). The consensus aminoacid sequence of the repetitive epitopes is shown in Fig. 1.

Cytoplasmic Repetitive Antigen (CRA)
LYS ALA ALA GLU ALA THR LYS VAL ALA GLU ALA GLU LYS GLN
Flagellar Repetitive Antigen (FRA)
MET GLU GLN GLU ARG ARG GLN LEU LEU GLU LYS ASP PRO ARG ARG
ASN ALA LYS GLU ILE ALA ALA LEU GLU GLU SER MET ASN ALA ARG
ALA GLN GLU LEU ALA ARG GLU LYS LYS LEU ALA ASP ARG ALA PHE
LEU ASP GLN LYS PRO GLU ARG VAL PRO LEU ALA ADP VAL PRO LEU
ASP ASP ASP SER ASP PHE VAL ALA

Figure 1 - Consensus aminoacid sequence of the repeat unit of CRA and FRA

It is interesting to note that most of the genes screened from *T. cruzi* expression libraries display various copies of repetitive motifs, indicating that repetitive epitopes are highly antigenic. Accordingly, other groups described antigens similar to CRA (Ibanez et al., 1988; Hoft et al., 1989; Levin et al., 1989) and to FRA (Ibanez et al., 1988; Levin et al., 1989; Cotrim et al., 1990). Despite the ubiquitous nature of these antigens, CRA and FRA are highly polymorphic in *T. cruzi* (Krieger et al., 1990). This polymorphis can be seen at the genomic level, where distinct restriction fingerprints are obtained for CRA and FRA in different *T. cruzi* strains.

Use Of Recombinant Antigens In Chagas' Disease Diagnosis

Although polymorphic in different strains, these antigens were recognized in the form of B-galactosidase fusion proteins by more than 95% of chagasic sera when individually tested using a radioimmunoassay procedure. This indicated that the antigenic determinants are well conserved. However, our first attempts to use these recombinant antigens in an ELISA test showed that some non-chagasic sera displayed a borderline response, very likely due to their reactivity with the B-galactosidase portion of the fusion proteins. In order to circumvent this problem, we adopted a strategy which consisted of expressing these recombinant antigens in the pMSgt11 vector (Scherf et al, 1990). This vector contains a cleavage site for factor Xa in the cloning site, hence allowing the enzymatic cleavage of the fusion protein with the subsequent release of the B-galactosidase. We first tested CRA expressed in pMSgt11 and the results were highly satisfactory: in a multi-center study carried out by the World Health Organization and involving nine laboratories and 24 antigens, it was concluded that CRA ranked as the best individual reagent (Moncayo & Luquetti, 1990).

However, a doubtful response was observed for some sera, as a result of reactivity too close to the cut-off serum control. When a mixture of CRA and FRA was used instead of the individual antigen, it became evident that some of the doubtful sera were in fact positive for Chagas' disease. Consequently, in order to improve the ELISA test we started to use a mixture (1:1) of CRA and FRA, resulting in a highly specific diagnosis reagent (Almeida et al., 1990; Krieger et al., in preparation).

The CRA+FRA ELISA was then tested using sera from different endemic regions, sera from patients bearing diseases which present cross-reactivity with Chagas' disease, and negative sera from endemic areas and from blood banks. The results were compared to those obtained with conventional serological tests (haemagglutination and indirect immunofluorescence), and to those obtained using an ELISA test consisting of a cytosolic extract (CYTO) of *T. cruzi* as antigen source. It was observed that the CRA+FRA ELISA, in addition of recognizing all tested chagasic sera, did not react with sera from patients bearing other diseases. On the contrary, both the Cyto ELISA (Table I) and the conventional serology tests (Table II) gave some false positive responses.

TABLE I

Comparision of CRA + FRA and CYTO ELISA with different human sera

Sera	CRA + FRA	CYTO
Chagasic(n=221)	221	221
Negative[1] (n=193)	0	0
Negative[2] (=49)	0	1
Schistosomiasis (n=15)	0	2
Malaria (n=12)	0	2
Syphilis (n=14)	0	4
Leishmaniasis (n=21)	0	3

1 - Negative sera from endemic areas
2 - Negative sera from blood bank

TABLE II

Comparision of the reactivity of conventional serological methods and CRA + FRA ELISA with sera which cross-react with Chagas' disease

Sera	IHA	IFI	CRA + FRA
Visceral Leishmaniasis (n=5)	5	5	0
Cutaneous leishmaniasis (n=5)	2	3	0
Leprosy (n=2)	0	2	0
Lupus (n=8)	0	2	0

CONCLUSIONS AND PERSPECTIVES

The data discussed above indicate that the use of *T. cruzi* recombinant antigens in an ELISA test provides a safe and accurate diagnosis for Chagas' disease. The main advantage of the recombinant ELISA is the fact that it gives very few (if any) false positive results in comparison to other reagents and methods frequently used for the diagnosis of the disease. These false positive responses should be avoided in virtue of the social problems they can cause for the patient. In addition, recombinant antigens are cheaper to produce than antigens isolated from the parasite, and this should have a direct impact on the price of the diagnostic reagent. In the particular case of the FRA and CRA antigens, their repetitive epitope structure suggests that synthetic peptides might be used in diagnosis in a near future. Indeed, we have recently tested a synthetic CRA peptide and the results showed that 65% of the tested chagasic sera were detected in ELISA. However, further investigations are necessary in order to determine whether this observed diminution in sensitivity was due to a technical problem related to the binding of the peptide to the ELISA plate or, alternatively, whether the problem was related to a poor exposition of the correct antigenic determinants.

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