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# USE OF RECOMBINANT ANTIGENS FOR THE DIAGNOSIS OF CHAGAS DISEASE AND BLOOD BANK SCREENING

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## INTRODUCTION

The diagnosis of Chagas' disease is still a problem because of difficulties related to the specificity and sensitivity of the available tests. This is an important point since every year there are many new cases of Chagas disease acquired by blood transfusion.

Most of the methods routinely used for the diagnosis of Chagas' disease are based on the detection of antibodies against *Trypanosoma cruzi* in the sera of patients. As a consequence, the accuracy of the diagnosis will depend on the specificity, availability and purity of the antigens used. The use of recombinant antigens should be of great help in improving the diagnosis of Chagas' disease, since the expression of suitable (specific) antigens might facilitate their production in large amounts, and with a acceptable degree of purity.

Recent work in several laboratories resulted in the cloning of several *T. cruzi* genes (Peterson et al., 1986; Dragon et al., 1987; Lafaille et al., 1987, 1989; Ibanez et al., 1988; Beard et al., 1988; Hoft et al., 1989; Van Voorhis & Eisen, 1989; Levin et al., 1989;), paving the way for the use of recombinant antigens in the diagnosis of Chagas' disease. The results presented below relate to the development of diagnostic reagents for Chagas' disease using two recombinant antigens partially characterized in our laboratory.

## DESCRIPTION OF THE ANTIGENS

The screening of a *T. cruzi* genomic expression library with trypomastigote and chagasic sera resulted in the selection of two clones which, in addition, did not react with non-chagasic sera (Lafaille et al., 1987). Partial nucleotide sequencing showed that these genes have a repetitive epitope structure: one of the clones consisted of a 14 amino acid repeat and the corresponding protein was located in the cytoplasm of the parasite (hence, cytoplasmic repetitive antigen or CRA); the other antigen consisted of a 68 amino acid repetitive unit and was located at the portion of the flagellum that faces the body of the parasite (hence, flagellar repetitive antigen or FRA) (Lafaille et al., 1989). The study of the expression of these antigens showed that FRA is constitutively expressed in epimastigotes and metacyclic trypomastigotes whereas CRA is not expressed in metacyclic forms (Krieger et al., 1990). In addition, these antigens are highly polymorphic in all *T. cruzi* strains tested (Krieger et al., 1990).

## USE OF CRA IN CHAGAS' DISEASE DIAGNOSIS

We first tested CRA in a radioimmunoassay (RIA), where the partially purified antigen was bound to a nylon membrane. The results showed a 92% overall agreement with conventional serology (IHA and IIF) data. However, some false positives were obtained, very likely due to recognition of the B-galactosidase portion of the fusion protein by some sera. This problem was partially solved by the densitometric analysis of the autoradiograms which contained three slots (corresponding to *T. cruzi* extract, CRA and B-galactosidase) reacting with each sera. However, the RIA is not appropriate for the screening of a large number of samples.

We then developed an ELISA. In order to avoid false positive responses against the B-galactosidase portion of the fusion protein, CRA was expressed in the pMS vector (Scharf et al., 1990) which contains a cleavage site for

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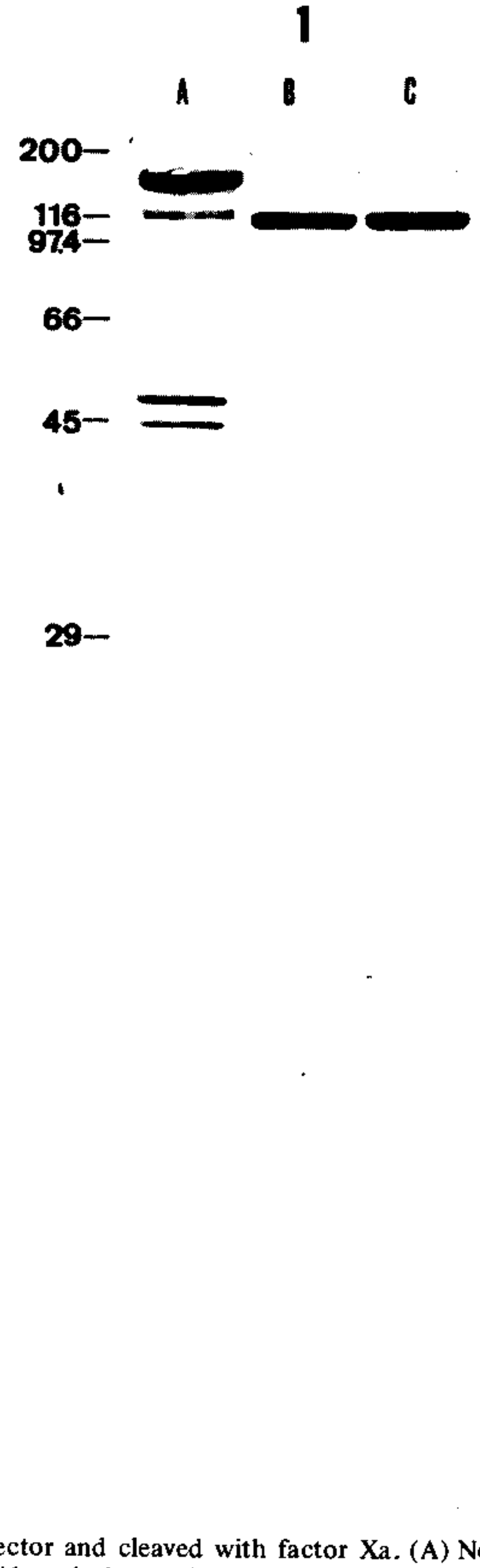


Analysis of CRA expressed in pMS vector and cleaved with factor Xa. (A) No digestion; (B) 24 h digestion and (C) 48 h digestion. (1) Polyacrylamide gel electrophoresis; (2) Western blot analysis using a B-galactosidase antiserum and (3) Western blot analysis using a CRA antiserum.

factor Xa. The Figure shows the kinetics of CRA digestion with factor Xa and western blot analysis with CRA and B-galactosidase antisera. This ELISA was used for the evaluation of the sera from the WHO Multicentre study, and 4 out of 50 tested sera were considered doubtful. In fact, using other serum samples we observed that some displayed an optical density in ELISA which was higher than the mean value of the negatives, but lower than the estimated cut-off. This was very likely due to a low titer response of some sera to CRA, and may also have been the case for the WHO Multicentre Study sera.

Similar results were obtained when FRA was used. Statistical analysis of the data obtained with these antigens indicated that there was no

TABLE I

Comparison of CRA + FRA and *Trypanosoma cruzi* cytosolic extract ELISA

| Sera extract | CRA + FRA Positive | CRA + FRA Negative | Cytosolic Positive | Cytosolic Negative |
|---|---|---|---|---|
| Chagas positive from Brazil northeast endemic area (n = 104) | 104 | 0 | 104 | 0 |
| Chagas negative from Brazil northeast endemic area (n = 138) | 0 | 138 | 0 | 138 |
| Chagas positive from Brazil southeast endemic area (n = 117) | 117 | 0 | 117 | 0 |
| Chagas negative from Brazil southeast endemic area (n = 55) | 0 | 117 | 0 | 117 |
| Negative from blood bank (n = 49) | 0 | 49 | 1 | 48 |
| Rheumatoid factor (n = 8) | 0 | 8 | 0 | 8 |
| Schistosomiasis (n = 15) | 0 | 15 | 2 | 13 |
| Malaria (n = 12) | 0 | 12 | 2 | 10 |
| Toxoplasmosis (n = 10) | 0 | 10 | 0 | 10 |
| Syphilis (n = 14) | 0 | 14 | 4 | 10 |
| Leishmaniasis (n = 21) | 0 | 21 | 3 | 18 |

TABLE II

Comparison of CRA + FRA ELISA and commercial ELISA kits with regard to their specificity against blood bank samples which agree in their IIF and IHA data for Chagas' disease

| IIF and IHA test | | ELISA test CRA + FRA | A | B | C | D |
|---|---|---|---|---|---|---|
| Positive (n = 111) | | 111 | 111 | 111 | 111 | 109 |
| Negative (n = 102) | | 102 | 102 | 102 | 102 | 104 |
| Total | 213 | 213 | 213 | 213 | 213 | 213 |

genetic restriction between CRA and FRA. Indeed, we observed that some sera displayed a negative response for the individual antigens and a positive response for a mixture of the two. This observation led us to use a mixture of CRA + FRA in ELISA.

USE OF CRA + FRA IN CHAGAS' DISEASE DIAGNOSIS

We tested the ELISA using a mixture of CRA + FRA with 543 human sera and compared its performance with an ELISA using a *T. cruzi* strain Y cytosolic extract as an antigen source. The samples consisted of positive and negative chagasic sera from two endemic regions, negative sera from a blood bank and sera negative for Chagas' disease but positive for other diseases which can present cross-reactivity with *T. cruzi*. The results are presented in Table I and show that the ELISA using CRA + FRA displayed a sensitivity and specificity of 100%. On the other hand, the ELISA using *T. cruzi* cytosolic antigens was not specific, resulting in 12 false positives.

The CRA + FRA ELISA was also directly tested in the field, in the endemic areas of Santa Cruz and Virgem da Lapa (Minas Gerais, Brazil). The data obtained were in full agreement with clinical and conventional serology (IHA and IIF) data, with the advantage of taking only a couple of hours to perform.

USE OF CRA + FRA IN BLOOD BANK SCREENING

The screening of blood bank samples is of the utmost importance in view of the risks of transmission of Chagas' disease via blood transfusion. In the particular case of Brazil, this aspect is even more important if one considers that 5% of the population is chagasic,

TABLE III

Comparative analysis of different serological methods for Chagas' disease diagnosis with human blood bank sera positive for other diseases

| Patients sera | IHA | IFI | ELISA | | | | | Number of sera |
|---|---|---|---|---|---|---|---|---|
| | | | CRA + FRA | A | B | C | D | |
| Syphilis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Hepatitis B | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 5 |
| AIDS | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Leprosy | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| Lupus | 0 | 2 | 0 | 1 | 1 | 1 | 0 | 8 |
| Malaria | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 10 |
| Cutaneous Leishmaniasis | 2 | 3 | 0 | 1 | 3 | 3 | 1 | 5 |
| Visceral Leishmaniasis | 5 | 5 | 0 | 0 | 5 | 5 | 3 | 5 |
| Total | 7 | 13 | 0 | 3 | 9 | 9 | 4 | |

and that many (if not most) of the blood donors belong to the population of immigrants from the rural region. In addition, it is important to consider the final cost of the blood since some samples require several different evaluations and are sometimes discarded without any real necessity due to false positives obtained in tests.

The CRA + FRA ELISA was tested in the blood bank from Fundação Hemocentro (São Paulo, Brazil) and the results were compared to those obtained with four commercial kits available in Brazil. The first assay consisted of testing sera samples that agreed in conventional serology (IHA and IIF) data. The results are presented in Table II and show that all the kits presented 100% specificity and, with the exception of kit D (98.2%), all of them displayed 100% sensitivity.

However, in a different test, when sera from patients bearing other diseases were tested, using different serological methods (IIF, IHA and ELISA), CRA + FRA was the only one that did not give rise to false positive responses, as shown in Table III. This indicates that although all assays can be useful for blood bank screening, only the CRA + FRA ELISA provides a safe diagnosis of Chagas' disease.

It is worth mentioning some preliminary data from a study which is being conducted using 77 sera from the Fundação Hemocentro in São Paulo, which do not agree on the basis of IHA and IIF data. The evaluation of these sera using the five different ELISA tests indicated that 36 of them (46.75%) did not react with any of the kits, and are very likely negative for Chagas' disease. On the other hand, 41 sera (53.25%) reacted with at least one of the ELISA tests, and from these 14 samples (18.18%) displayed a reactivity with the CRA + FRA ELISA. Those positive for CRA + FRA were also positive for at least two other tests. The clinical investigation of these patients is presently underway.

## CONCLUDING REMARKS

The results presented here indicate that we have made significant progress towards the development of a diagnostic kit for Chagas' disease with an ELISA using *T. cruzi* recombinant antigens. It is interesting to observe that differences can be obtained when the same antigen is tested in different laboratories (cf. the Multicentre Study from WHO in this issue). This is very likely due to the purity of the antigen and/or to the diagnostic procedure used.

The use of combined antigens gave better results than the use of individual antigens, since some sera can display a low titer response for individual antigens. However care should be taken in the choice of the antigens since some of them can display cross-reactivity with other diseases.

It is worth mentioning that the ELISA developed in our laboratory is suitable for the screening and diagnosis of a large number of samples, with applicability in blood banks and

in the field. This should render these procedures faster, cheaper and more reliable.

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REFERENCES

BEARD, C. A.; WRIGHTSMAN, R. A. & MANNING, J. E., 1988. Stage and strain specific expression of tandemly repeat 90 KDa surface antigen family in *Trypanosoma cruzi. Mol. Biochem. Parasitol., 28:* 227-234.

DRAGON, E. A.; SIAS, S. R.; KATO, E. A. & GABE, J. D., 1987. The genome of *Trypanosoma cruzi* contains a constitutively expressed, tandemly arranged multicopy gene homologous to a major heat shock protein. *Mol. Cell. Biol., 7:* 1271-1275.

HOFT, D. F.; KIN, K. S.; OTSU, K.; MOSER, D. R.; YOST, W. J.; BLUMIN, J. H.; DONELSON, J. E. & KIRCHHOFF, L. V., 1989. *Trypanosoma cruzi* expresses diverse repetitive protein antigens. *Infect. Immun., 57:* 1959-1967.

IBANEZ, C. F.; AFFRANCHINO, J. L.; MACINA, R. A.; REYES, M. B.; LEGUIZAMON, S.; CAMARGO, M. E.; ASLUND, L.; PETTERSON, U. & FRASCH, A. C. C., 1988. Multiple *Trypanosoma cruzi* antigens containing tandemly repeated aminoacid sequence motifs. *Mol. Biochem. Parasitol., 30:* 27-34.

KRIEGER, M. A.; SALLES, J. M.; ALMEIDA, E.; LINSS, J.; BONALDO, M. C. & GOLDENBERG, S., 1990. Expression and polymorphism of a *Trypanosoma cruzi* gene enconding a cytoplasmic repetitive antigen. *Exp. Parasitol., 70:* 247-254.

LAFAILLE, J. J.; LINSS, J.; CARDOSO, M. A. B.; KRIEGER, M. A.; AYMERICH S. & GOLDENBERG, S., 1987. Cloning of *Trypanosoma cruzi* stage specific genes. *Mem. Inst. Oswaldo Cruz, 82:* 252-259.

LAFAILLE, J. J.; LINSS, J.; KRIEGER, M. A.; SOUTO-PADRON, T: DE SOUZA, W. GOLDENBERG, S., 1989. Structure and expression of two *Trypanosoma cruzi* genes enconding antigenic proteins bearing repetitive epitopes. *Mol. Biochem. Parasitol., 35:* 127-136.

LEVIN, M. J.; MESRI, E.; BENAROUS, R.; LEVITUS, G.; SCHIJMAN, A.; LEVY-YEYATI, P.; CHIALE, P.; RUIZ, A. M.; KAHN, A.; ROSENBAUM, M.; TORRES, H. N. & SEGURA, E. L., 1989. Identification of major *Trypanosoma cruzi* antigenic determinants in chronic Chagas' heart disease. *Am. J. Trop. Med. Hyg., 41:* 530-538.

PETERSON, D. S.; WRIGHTSMAN, R. A. & MANNING, J. E., 1986. Cloning of a major surface antigen gene of *Trypanosoma cruzi* and identification of a nonapeptide repeat. *Nature, 322:* 566-568.

SCHERF, A.; MATTEI, D. & SCHREIBER, M., 1990. Parasite antigens expressed in *Eicherichia coli.* A refined approach for epidemiological analysis. *J. Immunol. Meth., 128:* 81-87.

VAN VOORHIS, W. C. & EISEN, H., 1989. A surface antigen of *Trypanosoma cruzi* that mimics mammalian nervous tissue. *J. Exp. Med., 169:* 641-652.