Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 78(4) :437-442, out./dez. 1983

EVI ANTIBODIES IN PATIENTS WITH CHAGAS' DISEASE: RELATIONSHIP WITH ANTI-*TRYPANOSOMA CRUZI* IMMUNOGLOBULINS AND EFFECTS OF SPECIFIC TREATMENT

Z. BRENER*
L.E. RAMIREZ*
A.U. KRETTLI**
J.R. CANÇADO***

Antibodies against heart vascular structures and striated muscle cells interstitium (EVI antibodies) persist in Chagas' disease patients who had been cured by specific treatment as demonstrated by negative xenodiagnosis, conventional serology (CS) and complement mediated lysis (CoML). On the other hand, EVI antibodies are either present or absent in treated patients presenting positive CS but negative CoML. Since CoML detects antibodies associated to resistance, EVI antibodies are not likely to participate in the control of T. cruzi *infections although they might be induced by cross-reacting antigens of heart cells and the parasite. They are neither necessarily related to antibodies responsible for CS. Absorption with* T. cruzi *and heart tissue confirms the suggestion that EVI antibodies are induced by a number of antigenic determinants, most from heart structures with a minor participation of* T. cruzi *antigens.*

Antibodies directed against endothelial cells, vascular structures and heart muscle (EVI antibodies) are highly prevalent in Chagas' disease patients (Cossio et al., 1974; Szarfman et al., 1977; Peralta et al., 1982). Although no final demonstration of a correlation between EVI antibodies and pathology has been provided, there has been suggested that these antibodies are induced by cross-reacting antigens between *T. cruzi* and host striated muscle cells. In this paper we investigated EVI antibodies in patients submitted to specific treatment, including some considered as parasitologically cured. In addition, we studied the relationship of EVI antibodies with the immunoglobulins responsible for conventional serological tests as well as those involved in the resistance against *T. cruzi* (Krettli & Brener, 1982; Krettli, Cançado & Brener, 1982).

Supported by the National Research Council, Brazil.

*Centro de Pesquisas René Rachou – FIOCRUZ, Caixa Postal 1743, 30000 Belo Horizonte, Minas Gerais, Brazil.

**Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais.

***Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais.

Received for publication March 10th and accepted April 4th, 1983.

MATERIAL AND METHODS

Chagas' disease patients – sera of the following groups were used: a) 8 untreated patients with positive indirect immunofluorescence test (IFT), complement fixation test (CFT) and complement mediated lysis (CoML); b) 5 patients submitted to a long-term treatment with N. benzyl-2-nitro-1-imidazolacetamide (benznidazol) and displaying post-therapeutic persistent negative IFT, CFT and CoML. Those patients (two recent chronic and three acute cases) are considered as cured on grounds of clinical and serological criteria (Krettli, Cançado & Brener, 1982); c) 4 patients treated with benznidazol who presented after treatment a dissociation of antibodies, namely, negative CoML and positive conventional serology (IFT and CFT).

Normal controls – sera from 8 normal individuals in which Chagas' disease has been excluded by epidemiological data and negative IFT, CFT and CoML were used.

Serological tests – the IFT was carried out according to Camargo (1966), using formalin-fixed epimastigotes obtained from acellular medium and fluorescein-conjugated rabbit anti-human IgG (Pasteur Institute). The CFT was performed according to the technique described by Freitas & Almeida (1949).

Complement-mediated lysis – anti-living blood trypomastigotes antibodies (ALBA) were investigated by the technique described by Krettli, Weisz-Carrington & Nussenweig (1979) using bloodstream forms of the *T. cruzi Y* strain collected from X-irradiated mice (650r).

EVI antibodies – mouse heart sections were used as substrate for the reactions. The organ was frozen at $-20^{o}C$ and 2 micra sections obtained in a cryostat (International Harris Cryostat). The unfixed sections were washed with PBS, pH 7.2 and treated with the diluted sera for 30 min at $37^{o}C$; after washing the material was further incubated for 30 min at $37^{o}C$ with fluoresceinated sheep anti-human IgG. The sections were washed, dried, mounted in buffered glycerin and examined in a Wild-Leitz Ortholux II fluorescense microscope. Only sera inducing the pattern described by Cossio et al. (1974) of reactions with endocardium, vascular structures and striated muscle were considered as positive for EVI antibodies.

Absorption test – 1 ml of the test sera diluted 4x was incubated for 4 hours at room temperature with 45 mg of lyophilized *T. cruzi* culture forms harvested from acellular medium; the suspension was centrifuged and the sera again incubated overnight at $4^{o}C$ with a new batch of 45 mg of lyophilized parasites. In some experiments sera were absorbed with heart tissue. Mouse hearts were removed, washed and after been minced, suspended in twice the volume of PBS, pH 7.2. Following homogenization in a tissue grinder the material was lyophilized and sera absorption performed according to the same schedule used for culture forms.

RESULTS

In the group of normal individuals, seven were negative and one positive for EVI antibodies. Titres of 1:128 and 256 were observed in two different occasions with the positive sera. In the group of 8 untreated chagasic patients, EVI antibodies were detected in 7, with titres ranging from 1:16 to 1:256.

Table I shows the results of CoML, conventional serology and EVI antibodies in the patients treated with benznidazol in which no evidence remained of an active infection as demonstrated by negativation of the serological tests (which were positive before treatment) and absence of anti-living blood forms antibodies (ALBA) detectable by the CoML. In all those cases, however, EVI antibodies were present in rather high titres.

TABLE I

EVI antibodies in patients with Chagas' disease treated with Benznidazol and considered as cured (serology and CoML negative)

Patient	*CoML*	*IFT*	*CFT*	*EVI antibodies*	*Months after treatment*
ARB	Neg.	Neg.	Neg.	1:128	36
ARB	Neg.	Neg.	Neg.	1:32	46
ARB	Neg.	Neg.	Neg.	1:32	58
CMDR	Neg.	Neg.	Neg.	1:256	3
CMDR	Neg.	Neg.	Neg.	1:256	7
CMDR	Neg.	Neg.	Neg.	1:256	13
NRT	Neg.	Neg.	Neg.	1:64	10
NRT	Neg.	Neg.	Neg.	1:256	21
BFF	Neg.	Neg.	Neg.	1:64	36
ZKS	Neg.	Neg.	Neg.	1:256	12

CoML = Complement mediated lysis
IFT = immunofluorescence test
CFT = complement-fixation test

Table II shows the results of the search of EVI antibodies in a group of treated patients presenting dissociation of antibodies, namely, with negative CoML and positive conventional serology. Those results show that EVI antibodies do not depend on the presence of lytic antibodies detectable by CoML neither of antibodies involved in the diagnosis of Chagas' disease (Patient JFS).

TABLE II

EVI antibodies in patients with Chagas' disease treated with Benznidazol and presenting dissociation of antibodies (negative CoML and positive IFT)

Patient	*CoML*	*IFT*	*EVI antibodies*	*Months after treatment*
JFS	Neg.	1:160	Neg.	10
JFS	Neg.	1:80	Neg.	11
RV	Neg.	1:320	1:32	9
RV	Neg.	1:160	1:16	13
RV	Neg.	1:160	1:64	30
RPR	Neg.	1:160	1:64	72
FDV	Neg.	1:640	1:256	11

Table III describes the effect of absorption of six different sera with *T. cruzi* culture forms on the titres of immunofluorescence test and EVI antibodies. The complete absorption of the antibodies detectable by IFT has not abolished the EVI antibodies. The residual antibodies left after absorption with lyophilized parasites were absorbed by a further treatment with mouse heart extract. In two sera of chronic patients which had

been absorbed only with lyophilized heart extract the EVI antibodies reactions that were positive at titres of 1:64 became negative whereas the IFT positive at titres 1:256 remained positive at the same titres in both patients. The EVI antibodies present in a non-chagasic individual (AAB) remained unchanged after absorption with *T. cruzi* but, as expected, they disappeared after incubation with heart tissue.

TABLE III

Effect of absorption of the sera with *Trypanosoma cruzi* and heart extracts on the titres of EVI antibodies.

Patients	*CoML*	*IFT*	*IFT (ATc)*	*EVI*	*EVI (ATc)*	*EVI (AHm)*	*Observations*
AAA	Posit.	1:256	Neg.	1:32	Neg.	–	Chagasic
GDV	Posit.	1:256	Neg.	1:64	1:64	Neg.	Chagasic
GFS	Posit.	1:128	Neg.	1:256	1:32	Neg.	Chagasic
CMDR	Neg.	Neg.	–	1:256	1:256	1:32	Chagasic cured
NRT	Neg.	Neg.	–	1:64	1:32	Neg.	Chagasic cured
AAB	Neg.	Neg.	–	1:64	1:64	Neg.	Non-chagasic

ATc – absorption with *T. cruzi* culture forms
AHm – absorption with mouse heart extract

DISCUSSION

The high prevalence of EVI antibodies among Chagas' disease patients has suggested the existence of cross-reacting antigens between *T. cruzi* and heart or skeletal striated muscle structures (Cossio et al. .1974; Szarfman et al., 1974). Recent studies demonstrated that *T. cruzi* shares common antigens with laminin, an important component of basement membranes; moreover, circulating antibodies against laminin have been detected in *T. cruzi* infected hosts (Szarfman et al., 1982). Since the possibility exists that EVI antibodies are induced by those common antigens, we investigated sera from patients in which *T. cruzi* has been erradicated by specific chemotherapy. In our experiments, EVI antibodies persisted in five patients treated with benznidazol in which no evidence of residual infection could be obtained as demonstrated by the prolonged negativation of serological diagnostic tests and CoML. Those results agree with previous findings of Schmunis et al. (1978) and Schmunis (1978) who reported that EVI antibodies persisted in six acute cases who presented negative IFT after specific treatment, suggesting to the authors that "EVI antibodies may be self-perpetuated in the absence of infection".

We have also demonstrated persistence of EVI antibodies in treated chagasic patients presenting dissociation of antibodies (positive conventional serology and negative CoML). Our results show that EVI antibodies are not depending on the existence of antibodies responsible for the serological diagnostic tests. Moreover, we have now demonstrated for the first time that EVI antibodies (despite the possibility of being induced by *T. cruzi* and heart cells cross-reacting antigens) seem not to be the same immunoglobulins involved in resistance against this parasite. As recently reported (Krettli & Brener, 1982; Krettli, Cançado & Brener, 1982) lytic antibodies directed against living bloodstream forms and detectable by CoML are associated to resistance against *T. cruzi*. As here demonstrated, EVI antibodies were present in high titres in many treated patients in which lytic antibodies were absent. Another evidence that EVI antibodies are not associated or participating in resistance against *T. cruzi* blood forms was given by data with "antibody-dependent cytotoxicity (ADCC)" test in which only sera presenting

ALBA mediate destruction of the parasites (Martins et al., 1982). Sera from chagasic patients with high titres of EVI antibodies but devoided of lytic antibodies are unable to mediate destruction of blood forms through ADCC whereas EVI-negative sera displaying lytic activity clearly participate in the cytotoxicity reaction (our unpublished data).

The nature of the EVI antibodies is not yet fully understood. Absorption tests performed with different sera has shown that: a) *T. cruzi* culture forms which completely abolished positive IFT from chagasic sera (treated or untreated patients) did not remove entirely EVI antibodies; b) neither did the same absorption remove EVI from normal individuals; c) heart extract completely removed EVI antibodies from different sera (chagasic or non-chagasic) but not the antibodies responsible for the IFT reactions. Such findings apparently confirm the suggestion of Lenzi, Lenzi & Andrade (1982) that EVI antibodies are generated by many antigenic determinants, most of them derived from heart structures and only a small number from *T. cruzi.* This reasoning would explain the persistence of the EVI factor in chagasic patients parasitologically cured, the apparently higher efficiency of absorption of EVI antibodies with heart extracts than with *T. cruzi* culture forms and, finally, the fact that IFT remain positive in sera absorbed with heart tissue.

The presence of EVI antibodies in treated patients presenting positive conventional serology has an important practical implication. Since in chronic patients submitted to specific treatment xenodiagnosis becomes steadily negative whereas serological diagnostic reactions remain positive (review: Brener, 1979), there has been suggested that those patients might actually be cured and that the positive serology would be caused by heart cells antigens which cross-react with *T. cruzi.* Our demonstration that EVI antibodies are not related to antibodies involved in serology rules out this possibility.

Whether the persistence of EVI antibodies after curative treatment may influence the clinical course of the disease is still a matter of discussion. The role played by EVI antibodies in the pathogeny of Chagas' disease has not been completely demonstrated but Schmunis et al. (1978) listed a number of arguments supporting a pathological effect of this auto-immune response, namely, its higher prevalence in patients with cardiopathy than in assymptomatics, the presence of muscle-bound immunoglobulins in EVI-positive patients and the existence of skeletal muscle cells lesions associated to the presence of EVI antibodies. A prolonged follow-up of a large number of treated cases of Chagas' disease would be necessary to reach a reliable conclusion.

RESUMO

Anticorpos contra estruturas vasculares do coração e interstício de musculatura estriada (anticorpos EVI) persistem em pacientes com doença de Chagas curados por tratamento específico e que apresentam negativos o xenodiagnóstico, sorologia convencional (SC) e o teste de lise mediada por complemento (LMCo). Além disso, o anticorpo EVI pode estar presente ou não em pacientes tratados que apresentam SC positiva mas LMCo negativa. Como a LMCo detecta anticorpos associados à resistência, os anticorpos EVI provavelmente não participam do controle da infecção pelo *T. cruzi* (embora sejam induzidos por antígenos comuns a estruturas cardíacas e ao parasita). Os anticorpos EVI não são também necessariamente relacionados aos anticorpos responsáveis pela SC. Experiências de absorção com *T. cruzi* e tecido cardíaco confirmam a sugestão de que esses anticorpos são induzidos por vários determinantes antigênicos, a maioria dos quais de tecido cardíaco mas com menor participação de antígenos do *T. cruzi.*

REFERENCES

BRENER, Z., 1979. Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Western Hemisphere. *Pharmac. Ther.* 7 :71-90.

CAMARGO, M., 1966. Fluorescent antibody test for serodiagnosis of American Trypanosomiasis. Technical modification employing preserved culture forms of *Trypanosoma cruzi* in a slide test. *Rev. Inst. Med. trop. São Paulo 8* :227-234.

COSSIO, P.M.; LAGUENS, R.P.; DIEZ, C.; SZARFMAN, A.; SEGAL, A.; ARANA, R.M., 1974. Chagasic cardiopathy. Antibodies reacting with plasma membrane of striated muscle and endothelial cells. *Circulation 50* :1252-1259.

FREITAS, J.L.P. & ALMEIDA, J.O., 1949. Nova técnica para fixação do complemento para moléstia de Chagas. Reação quantitativa com antígeno gelificado de culturas de *Trypanosoma cruzi. O Hospital* (Rio de Janeiro) *35* :787-800.

KRETTLI, A.U. & BRENER, Z., 1982. Resistance against *Trypanosoma cruzi* associated to anti-living trypomastigote antibodies. *J. Immunol. 128* :2009-2012.

KRETTLI, A.U.; CANÇADO, J.R. & BRENER, Z., 1982. Effect of specific chemotherapy on the levels of lytic antibodies in Chagas' disease. *Trans. Roy. Soc. Trop. Med. Hyg. 76* :334-340.

KRETTLI, A.U.; WEISZ-CARRINGTON, P. & NUSSENZWEIG, R.S., 1979. Membrane-bound antibodies of bloodstream *Trypanosoma cruzi* in mice: strain differences in suscetibility to complement-mediated lysis. *Clin. Exper. Immunol. 37* :416-423.

LENZI, H.L.; LENZI, J.G.A.; ANDRADE, Z.A., 1982. Experimental production of EVI antibodies. *Am. J. Trop. Med. Hyg. 31* :48-52.

MARTINS, M.V.C.L.; SANCHEZ, G.P.; KRETTLI, A.U. & BRENER, Z., 1982. Antibody-dependent cellular citotoxicity (ADCC) with sera from mice chronically infected or immunized with *Trypanosoma cruzi.* IX Reunião Anual sobre Pesquisa Básica em Doença de Chagas, Caxambu, pp 63.

PERALTA, J.M.; MANIGOT, D.A.; MUSCELLI, E.O.A.; MAGALHÃES, T.C.R.; ALMEIDA, E.A. & BASTOS, A., 1982. Anticorpos EVI e NP na infecção chagásica crônica em pacientes com diferentes formas clínicas. *Rev. Inst. Med. trop. S. Paulo 24* :6-10.

SCHMUNIS, G.A., 1978. A resposta imune humoral na infecção humana recente pelo *Trypanosoma cruzi.* Thesis. Universidade Federal do Rio de Janeiro, 96 pp.

SCHMUNIS, G.A.; COSSIO, P.M.; SZARFMAN, A.; COARASA, L. & ARANA, R.M., 1978. Tissue-reacting antibodies (EVI antibodies) in nifurtimox-treated patients with Chagas' disease. *J. Infect. Dis. 138* :401-404.

SZARFMAN, A.; COSSIO, P.M.; DIEZ, C.; ARANA, R.M. & SADUN, E., 1974. Antibodies against endocardium, vascular structures and interstitium of striated muscle that cross-react with *T. cruzi* and *T. rhodesiense. J. Parasitol. 60* :1024.

SZARFMAN, A.; COSSIO, P.M.; SCHMUNIS, G.A. & ARANA, R.M., 1977. The EVI antibody in acute Chagas' disease. *J. Parasitol. 63* :149.

SZARFMAN, A.; TERRANOVA, V.P.; RENNARD, S.I.; FOIDART, J.M.; LIMA, M.F.; SCHEINMAN, J.I. & MARTIN, G.R., 1982. Antibodies to laminin in Chagas' disease. *J. Exp. Med. 155* :1161-1171.