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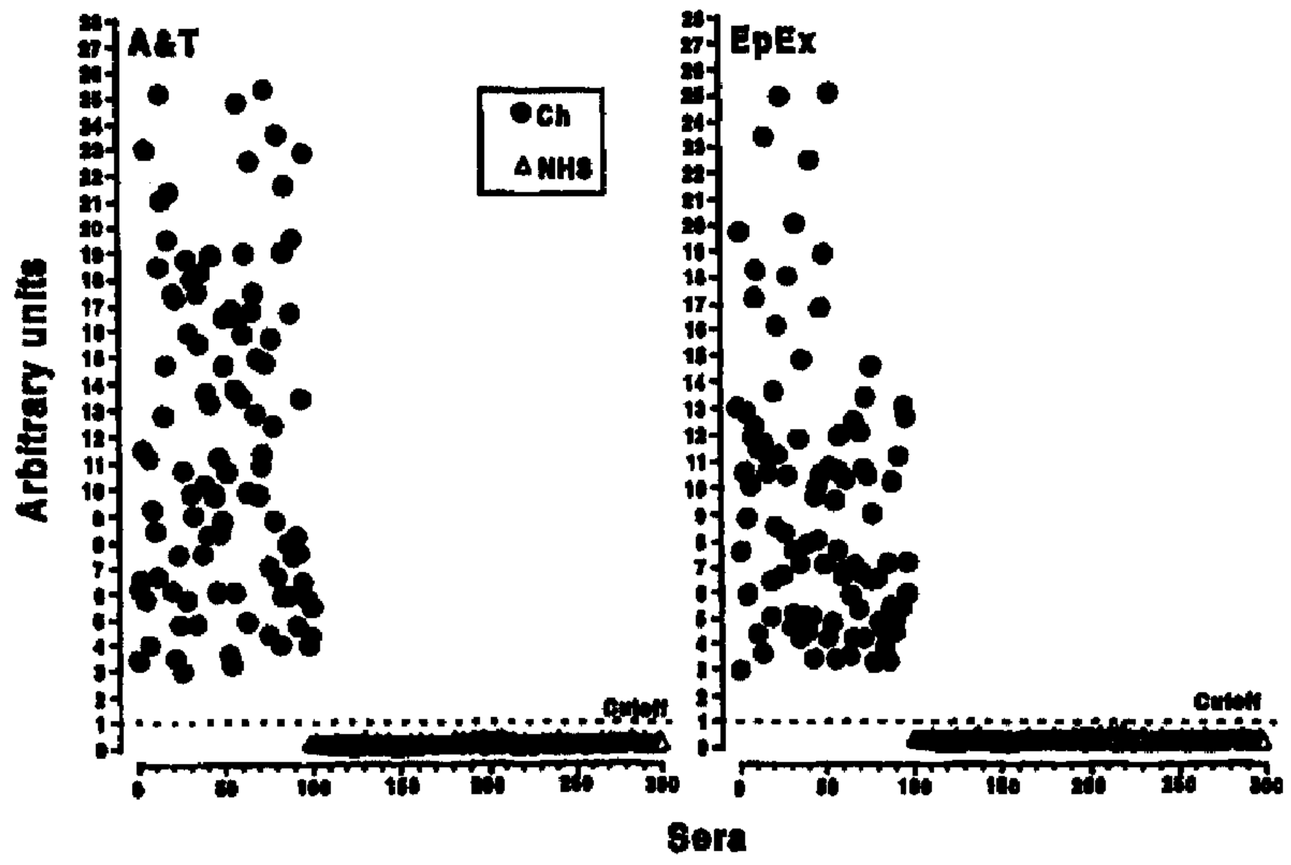
(54) **DIAGNOSTIC SEROLOGIQUE DE LA MALADIE DE CHAGAS**
(54) **SEROLOGICAL DIAGNOSIS OF CHAGAS' DISEASE**



**Evaluation of the sensitivity of CL - ELISAs using A&T and EpEx antigens.
Ch, chronic Chagasic sera (n=100); NHS, normal human sera (n=200).**

(57) L'invention concerne un procédé par chimiluminescence (CL)-ELISA avec des antigènes purifiés et complexes de Trypanosoma cruzi permettant de diagnostiquer de manière spécifique et sensible la maladie de Chagas chez des patients et dans des échantillons sanguins. Les préparations utilisées sont un antigène spécifique trypomastigote (A & T) et un extrait d'épimastigote (EpEx) permettant de réguler la sensibilité. La forte sensibilité du procédé CL-ELISA permet une utilisation de quantité extrêmement petite d'antigène et une dilution de sérum dans des tests de routine de 1:2000, réduisant au minimum ainsi les

(57) A chemiluminescent (CL)-ELISA method with purified and complex antigens of Trypanosoma cruzi is proposed for the specific and sensitive diagnosis of Chagas' disease in patients and blood bank samples. A trypomastigote specific antigen (A & T) together with an epimastigote extract (EpEx), used as a control of sensitivity, are the preparations used. The high sensitivity of the CL-ELISA method permits the use of extremely small amounts of antigen and allows a serum dilution in routine tests as high as 1:2,000, thus reducing the nonspecific or false-positive reactions to a minimum. The use of the A & T purified antigen

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eliminatesréactions non spécifiques ou faux-positifs. L'utilisation de l'antigène purifié A & T élimine les réactivités croisées avec d'autres agents d'infection, détecte l'infection active et permet de surveiller la chimiothérapie chez les patients chroniques. L'utilisation de la préparation antigène EpEx ne confirme pas seulement les résultats positifs avec A & T mais suggère, lors de divergences, d'autres infections telles que la leishmaniose. Comparé à d'autres tests courants utilisés dans les banques de sang principales, le procédé CL-ELISA avec les antigènes A & T et EpEx, testé en parallèle, s'est avéré nettement plus efficace en éliminant des résultats indéterminés ou en augmentant les statistiques d'échantillons positifs diagnostiqués.

cross-reactivities with other infectious agents, detects active infection, and serves to monitor chemotherapy in chronic patients. The use of the EpEx antigenic preparation not only confirms the positive results with A & T but also, in case of discrepancy suggests other infections such as leishmaniasis. In comparison with current tests used in major blood banks, the CL-ELISA method with A & T and EpEx antigens, tested in parallel, proved to be clearly superior either by eliminating indeterminate results or by increasing the statistics of diagnosed positive samples.

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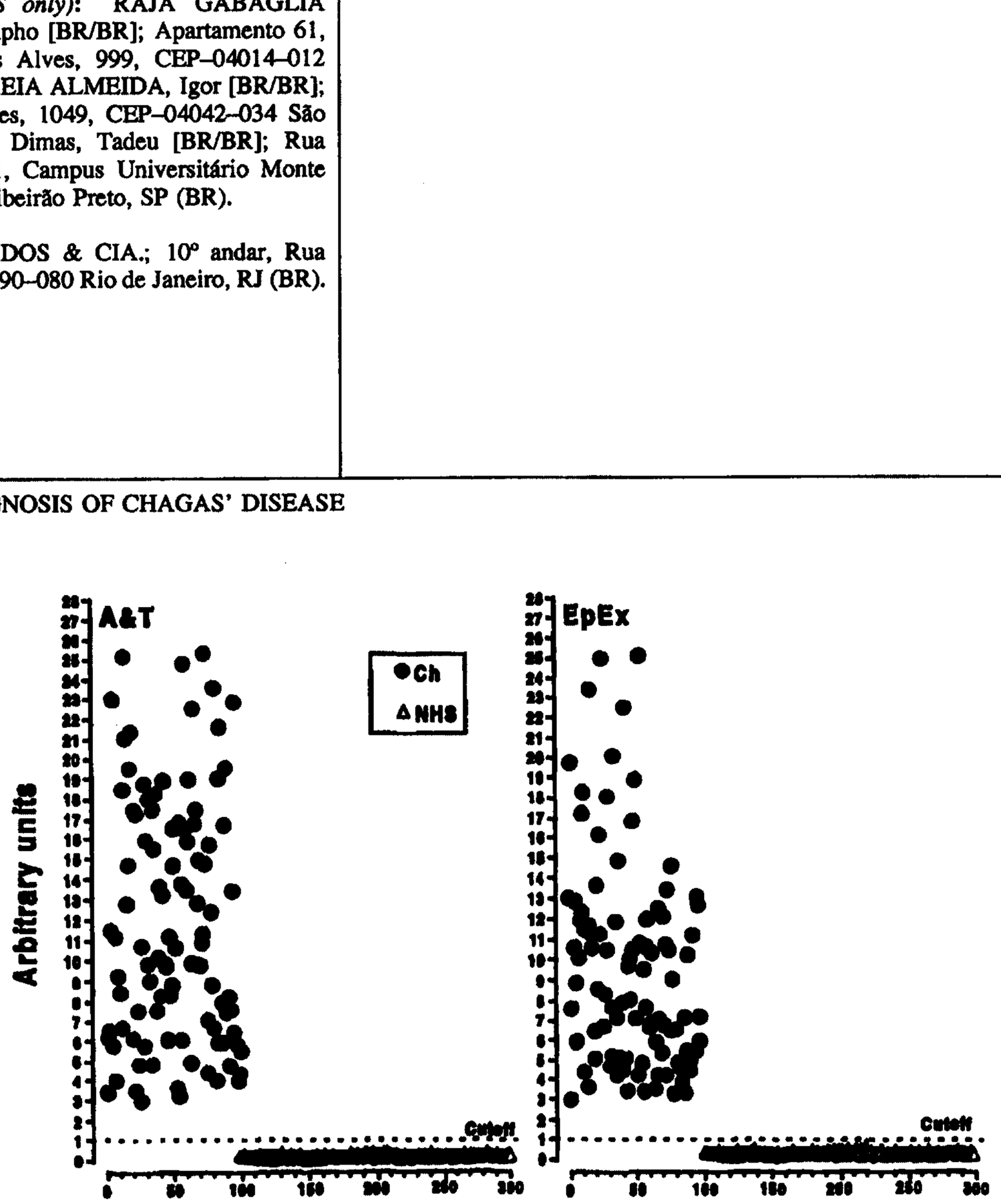
(54) Title: SEROLOGICAL DIAGNOSIS OF CHAGAS' DISEASE

(57) Abstract

A chemiluminescent (CL)–ELISA method with purified and complex antigens of *Trypanosoma cruzi* is proposed for the specific and sensitive diagnosis of Chagas' disease in patients and blood bank samples. A trypomastigote specific antigen (A & T) together with an epimastigote extract (EpEx), used as a control of sensitivity, are the preparations used. The high sensitivity of the CL–ELISA method permits the use of extremely small amounts of antigen and allows a serum dilution in routine tests as high as 1:2,000, thus reducing the nonspecific or false–positive reactions to a minimum. The use of the A & T purified antigen eliminates cross–reactivities with other infectious agents, detects active infection, and serves to monitor chemotherapy in chronic patients. The use of the EpEx antigenic preparation not only confirms the positive results with A & T but also, in case of discrepancy suggests other infections such as leishmaniasis. In comparison with current tests used in major blood banks, the CL–ELISA method with A & T and EpEx antigens, tested in parallel, proved to be clearly superior either by eliminating indeterminate results or by increasing the statistics of diagnosed positive samples.



**Evaluation of the sensitivity of CL - ELISAs using A&T and EpEx antigens.
Ch, chronic Chagasic sera (n=100); NHS, normal human sera (n=200).**

SEROLOGICAL DIAGNOSIS OF CHAGAS' DISEASE

Background of the Invention

Chagas'disease is characterized by a short-term acute phase, with very few clinical symptoms, and a long-term chronic phase, usually accompanied by severe gastrointestinal and/or cardic complications which result in permanent physical disability or death.

Chagas'disease is an endemic disease caused by the flagellate *Trypanosoma cruzi*. In Latin America, approximately 16 to 18 million individuals are already infected and as many as 90 million individuals are at risk of infection (W.H.O., 1991). The disease is transmitted in Nature by Triatominae vectors. As a result of effective public health measures for the control of the vector in most countries. blood transfusion is quantitatively the most important form of transmission of the disease today. In Latin America, blood samples with antibodies associated with Chagas'disease represent 1-4% of the total blood samples in major Hemocenters. More recently, Chagas'disease has also become a major public health concern in North America, owing to the increasing number of immigrants from Latin American countries, in the last decade. Recent studies estimate that there may be in the United States approximately 100,000 *Trypanosoma cruzi*-infected individuals with potential risk of transmitting Chagas'disease by blood transfusion (Hagar and Rahimtoola, 1991).

The diagnosis of acute Chagas'disease is not a problem because of the large number of parasites in the blood. In contrast, the chronic phase is diagnosed by serological methods because of the very small number or absence of circulating parasites. This has also restricted so far the use of polymerase chain reaction (PCR) with specific primers, as the final diagnostic test of Chagas' disease, before a major epidemiologic survey of sera from chronic patients is carried out. The three serological methods that are currently being used in blood banks - hemmagglutination (HA), indirect immunofluorescence (IIF) and enzyme-linked

immunosorbent assay (ELISA)- utilize mixtures of antigens prepared from the epimastigote form of the parasite. According to the World Health Organization (WHO), at least two positive tests of the three cited above are necessary for the diagnosis of the disease. Blood samples that are positive to only one of the three tests are classified as "indeterminate or inconclusive" and, in consequence, discarded. The indeterminate diagnosis is associated with 20 to 90% of all blood samples that gave one or more positive tests for Chagas'disease, depending on the methods employed and how they are applied. This high percentage of indeterminate results represents a serious problem in blood banks, both in terms of volume of discarded blood and doubtful diagnosis of Chagas'disease. In fact, a blood sample with a false positive test is no longer used for transfusion or isolation of cells and other blood components. Such loss of donated blood also affects the production of blood derivatives such as albumin, immunoglobulins and clotting factors which are of commercial value. Conversely, a blood sample with a false negative test is a dangerous source of contamination by the parasite.

The disadvantages of the current serological methods can be summarized as follows:

1. Low sensitivity: current methods use human sera at low dilutions, with a consequent increase in the background due to the cross-reactivity with natural antibodies and low-titer antibodies resulting from nonspecific polyclonal activation. Specific recombinant or synthetic epimastigote antigens, singly or in mixtures, are not sufficiently sensitive because they react only with a limited number of specific antibodies present in the sera of chronic Chagasic patient.

2. Low specificity: serological tests using epimastigote extracts cross-react with antigens from microbial sources other than *Trypanosoma cruzi*, notably *Leishmania* and some fungal and bacterial antigens.

Brief Summary of invention

The invention describes the purification of the A&T and EpEx antigens, and their use in a chemiluminescent enzyme-linked immunosorbent assay (CL-

ELISA), for the accurate diagnosis of Chagas'disease. When carried out in parallel, the results of the tests taken together provide high sensitivity and high specificity not obtainable with conventional methods described in the literature and/or which are commercially available.

Detailed description of the invention

Purification of the A&T antigen

A&T antigen is purified from trypomastigote forms of *Trypanosoma cruzi* according to Almeida *et al.*, 1993 and Almeida *et al.*, 1994a . Trypomastigote forms are obtained from infected green monkey kidney fibroblasts (LLC-MK_2 cells) cultured in Dulbecco's modified Eagle medium (D-MEM) containing 10% fetal bovine serum. The cell-derived trypomastigotes are collected 6-7 days later, following their release from infected cells, from the top fluid after sedimentation of the cell debris and incubation for 1.5 h at 37°C. Parasites are washed 3 times in 0.15 M phosphate-buffered saline (PBS), pH 7.4, centrifuged at 12,000*g*, and kept at -70°C until lyophilization. Lyophilized trypomastigotes are sequentially extracted 5 times with 10 volumes of chloroform/methanol (2:1), chloroform/methanol (1:2), chloroform/methanol/water (10:20:8), for 30 min each time, at room temperature. After centrifugation at 12,000*g*, the organic extracts are discarded and the final delipidated pellet is dried under a stream of nitrogen. The dry pellet is then extracted 5 times with 10 volumes of 9% 1-butanol for 2 h each time, at room temperature. The soluble extract corresponds to the fraction containing at A&T antigen together with some hydrophilic and hydrophobic contaminants. The A&T-containing fraction is then lyophilized for 24 h and chromatographed on a column of octyl-Sepharose (Pharmacia-LKB, Upsala, Sweden), pre-equilibrated with 5% 1-propanol in 0.1 M ammonium acetate buffer, pH 7.2. The A&T-containing fraction dissolved in 5% 1-propanol in 0.1 M ammonium acetate buffer, pH 7.2 is applied to the column at a low flow rate. The colum is washed with 5% 1-propanol in 0.1 M ammoniun acetate buffer, pH 7.2 and eluted with a 1-propanol gradient (5-60%). The column fractions containing

the A&T antigen are tested for immunoreactivity with a specific polyclonal antibody generated against the A&T antigen (anti-A&T antibody). The A&T-positive fractions from the octyl-Sepharose column are pooled, dried and partitioned between water and 1-butanol. The aqueous phase is lyophilized for 24 h, resuspended in 5% 1-propanol in ammonium acetate 0.1 M, pH 7.2 and applied to the phenyl-Superose column (Pharmacia-LKB, Sweden) (pre-equilibrated with 5% 1-propanol in ammonium acetate 0.1 M, pH 7.2). The column is eluted with a 1-propanol gradient (5-60%). Material eluting in earlier fractions (column void) and containing the A&T antigen is pooled and lyophilized for 24 h. The material included in the column is basically constituted of hydrophobic contaminants, mainly phospholipids. Finally, as a final purification step to eliminate hydrophilic contaminants, A&T antigenic preparation is re-applied to a column of octyl-Sepharose (Pharmacia-LKB, Sweden), pre-equilibrated with 5% 1-propanol in 0.1M ammonium acetate buffer, pH 7.2. The A&T-containing fraction dissolved in 5% 1-propanol in 0.1 M ammonium acetate buffer, pH 7.2 is applied to the column at a low flow rate. The column is washed with 5% 1-propanol in 0.1 M ammonium acetate buffer, pH 7.2 and eluted with a 1-propanol gradient (5-70%) and eluted with a shallow 1-propanol gradient (20-40%). The fractions are assayed for immunoreactivity with the anti-A&T antibody by dot-blotting and Western-blotting. Antibody binding fractions are pooled, exhaustively dialyzed against deionized water, lyophilized for 48 h, redissolved in deionized water and stored at -70°C.

<u>Purification of the EpEx antigen</u>

EpEx antigen is prepared from epimastigote forms of *Trypanosoma cruzi*, Tulahuén strain. Parasites are cultured at 28°C, in Schneider's insect medium containing 20% fetal calf serum. After 7-10 days, the parasites are collected from the culture supernatant, washed three times with 100 mM phosphate-buffered saline, pH 7.4 and centrifuged at 12,000*g* for 30 min, at 4°C. Pelleted parasites are immediately resuspended in 10 mM Tris-HCl buffer, pH 7.5, 0.2 mM

leupeptin, 2 mM EDTA, 1% nonanoyl-N-methylglucamide (lysis buffer), and submitted to four cycles of freezing and thawing, in liquid nitrogen and water-bath (37°C), respectively. The resulting lysate is centrifuged at 10,000*g* for 5 min, at 4°C. The supernatant, containing the EpEx antigenic preparation, is removed and stored at -70°C.

Chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) using EpEx and A&T antigens

Chemiluminescent ELISA is carried out according to protocols previously described (Almeida *et al.*, 1993, 1994b). A&T (at 0.15µg dry weight/µl deionized water) and EpEx (at 0.15µg protein/µl of lysis buffer) antigens are diluted in 50 mM sodium carbonate buffer, pH 9.6, for a final concentration of 0.2 ng/µl and 0.8 ng/µl, respectively. Fifty microliters of each antigen are separately added to each well of milky-white 96-well Maxisorp FluoroNunc plates (Nunc, Denmark). After 12 h at 4°C, plates are washed 5 times with 0.15 M phosphate-buffered saline, pH 7.4, 0.05% Tween 20 (PBS-T) and blocked with 0.1% bovine serum albumin (BSA) in 50 mM sodium carbonate buffer, pH 9.6, for 12 h at 4°C or, alternatively, for 2 h at 37°C. Plates are then washed 5 times with 0.15 M phosphate-buffered saline, pH 7.4, 0.05% Tween 20 (PBS-T). The human sera, diluted 1:2,000 in PBS-T containing 0.5% BSA (PBS-TB), are added to the plates and incubated for 30 min at 37°C. Plates are washed 5 times with PBS-T, the excess liquid removed by inversion or filter paper, and then incubated with biotinylated goat anti-human IgG (Amersham, UK), diluted 1:2,000 with PBS-TB, for 30 min at 37°C. After washing 5 times with PBS-T, a streptavidin-horseradish peroxidase conjugate (Amersham, UK) diluted 1:1,000 with PBS-TB is added, following incubation for 30 min at 37°C. Plates are washed 5 times with PBS-T, the excess liquid removed by inversion on filter paper, and then incubated with luminol (ECL reagents, Amersham, UK), diluted 1:20 in 50 mM carbonate buffer, pH 9.6, for 1-5 min at room temperature. Thereafter, the reaction is quantified

using a luminometer for 96-well polysterene plate readings. The results are expressed as relative luminescent units (RLU). Cutoff values for A&T and EpEx CL-ELISAs were first calculated by determining the reactivities of 200 normal human sera (NHS). The mean and SD of these 200 reactivities were determined. A value of 10 times the SD was added to the mean for the cutoff value,.The dispersion of the RLU readings for 200 NHS showed SD very close to the means using both A&T and EpEx CL-ELISAs (143 ± 123 and 177 ± 151, respectively). Therefore, for each plate in which a single negative control (pool of 100 NHS) in quadruplicate was included, the cutoff values for A&T and EpEx CL-ELISA were established as 10 times the negative control mean minus the background control mean (cutoff value = 10 x negative control mean - background control mean).

Result interpretation:

To interpret the results obtained, the luminometer reading of a serum sample is divided by the predeterminated cutoff value. A positive result is defined when the relative serum reading (RLU) is greater than 1, which represents the cutoff value. Conversely, a negative sample has an RLU equal or lower than 1.

1. The high sensitivity of the chemiluminescent (CL)-ELISA method permits the use of high dilutions of sera (1:2,000) (Fig. 1), and thus eliminates most of the nonspecific or false-positive reactions of current methods, which use serum dilutions in the 1:30 to 1:400 range (Tables 1 and 2).

Table 1 - Comparison between CL-ELISA (with A&T and EpEx) and conventional diagnostic tests with a panel of sera with inconclusive serology for Chagas' disease.

Diagnostic test	Serum dilution	Number of sera			Number of sera	
		Negative	Inconclusive	Positive	False-Negative	False-Positive[1]
Chemiluminescence						
A&T-CL-ELISA[2]	1:2,000	74	0	26	0	0
EpEx-CL-ELISA	1:2,000	72	0	28	0	2
Western blotting-EpEx	1:400	72	11	17	0	2
ELISA-EpEx	1:100	77	7	16	3	0
Commercial kits						
ELISA-A1	1:41	10	70	20	0	64
ELISA-A2	1:41	41	21	38	0	33
ELISA-B	1:41	67	8	25	0	7
ELISA-C	1:41	69	17	14	0	5
HA	1:40	79	12	9	5	0
IF	1:30	79	6	15	5	0

[1] No. Of false-positive sera = [no. Of positive + inconclusive sera with each test] - no. Of positive sera with the reference method (A&T-CL-ELISA).

[2] CL-ELISA with A&T antigen (CLE-A&T) is considered the gold method.

Table 2 - CL-ELISA reactivity of A&T and EpEx antigens with inconclusive and heterologous sera.

Conventional serology	Number of sera	Conventional tests [1]	Reactive sera in CL-ELISA	
			A&T	EpEx
Inconclusive for Chagas disease	100	100	26	28[2]
Leishmaniasis				
visceral	11	5	0	2
cutaneous	16	10	0	1
Autolmmune diseases	30	0	0	0
Infectious diseases				
AIDS	24	0	0	0
Hepatitis	24	0	0	0
Syphilis	24	0	0	0
Paracoccidioidomycosis	5	1	0	0
Poli A/C vaccinated *(Neisseria meningitidis)*				
pre-immune	5	0	0	0
Immunized	5	5	0	0
Chagas' disease	100	100	100	100
Normal human sera	200	0	0	0

[1] Number of sera giving at least 1 positive reaction in the conventional Chagas' disease serology (indirect immunofluorescence, indirect hemagglutination and ELISA).

[2] Two reactions with EpEx are false-positive.

2. The A&T antigen is a purified preparation of closely related molecules thar are specific of the trypomastigote stage obtained in tissue culture of mammalian

cells, thus being very similar to the infective forms of the parasite that cause the disease in man.

3. Since the serological reactions with A&T antigen are highly specific, there is no cross-reactivity with antigens from a variety of other infectious agents including *Leishmania*, and with natural antibodies and low-titer antibodies resulting from nonspecific polyclonal activation (Table 2).

4. The A&T antigen is easily obtainable in amounts sufficient for a great number of tests in appropriate ELISA plates for chemiluminescent reading. Moreover, the purified A&T antigen is highly stable when fixed on plates for prolonged periods.

5. Since A&T antigen reacts with lytic (protective) antibodies, characteristic of active infection and present in high titers in chronic patient sera, it can be used to monitor the response of patients to chemotherapy (Fig. 2).

6. The EpEx complex antigen is prepared from the epimastigote form and contains many components that are also expressed in the infective stage. It reacts with antibodies that are recognized by conventional serology for Chagas'disease, but not with those antibodies whose reactions are due to artifacts such as blocking reagents, culture medium supplements, etc.

7. The EpEx antigen is readily prepared from fast growing epimastigote culture, and although it is not as specific as A&T purified antigen, it is highly sensitive and provide complementary and confirmatory data for the positive reactions obtained with A&T antigen (Fig. 1).

8. The advantages of using both A&T and EpEx antigens in parallel tests are the following:

a) the antigens present in both tests are highly sensitive and therefore, a positive result with both antigens provides a diagnosis with a high level of confidence (Fig. 1, Table 1);

b) positive reactivity with EpEx, and negative with A&T, while eliminating active Chagas' infection, suggests leishmaniasis or another infectious disease (Table 2);

c) a decrease in the reactivity with A&T followed by a decrease with EpEx has prognostic value, and is a criterion of cure in patients submitted to chemotherapy (Fig. 2);

d) when applied to sera classified as "indeterminate" (*i.e.* sera which are negative to one or two of the following tests: hemagglutination, immunofluorescence and ELISA), the A&T and EpEx tests provide unambiguous results, thereby eliminating inconclusive serological diagnosis of Chagas' disease (Tables 1 and 2)

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CLAIMS

1. Process of preparation and utilization of antigens of Trypanosoma Cruzi, characterized by the fact that EpEx antigen is prepared from epimastigote forms of T. Cruzi, Tulahuén strain, the parasites thereof having been cultured at a temperature of about 28°C in Schneider's insect culture medium containing about 20% fetal calf serum, and wherein after 7 – 10 days, the parasites are collected from the culture supernatant, washed 3 – 5 times with phosphate-buffered saline at about 100 mM, at a pH of 7.3 – 7.4 and centrifuged at 12,000 – 14,000g for 30 – 45 minutes, at 4 – 8°C and the parasites being thereafter immediately resuspended in a Tris-HCl buffer at about 10 mM, pH of 7.4 – 7.5, containing about o.2 – 0.4 mM leupeptin, about 2 – 3 mM ETDA, about 1% nonanoyl-N-methylglucamide, and being submitted to 4 – 6 cycles of freezing and thawing, in liquid nitrogen and water-bath (about 37°C), respectively, the resultant lysate being centrifuged at 10,000 – 12,000g for 5 – 10 minutes, at about 4 – 8°C, and the supernatant, containing the EpEx antigenic preparation, being removed and stored at –70°C until the time of use.

2. Process, according to in claim 1, characterized by the the fatc that the pH of the phosphate-buffered saline is 7.4.

3. Process, according to claim 1 or 2, characterized by the fact that the washed parasites are centrifuged at 12,000g for 30 minutes, at 4°C.

4. Process, according to claims 1 to 3, characterized in that the Tris-HCl buffer has a pH of 7.5, with 0.2 mM leupeptin, 2 mM ETDA, and 1% nonanoyl-N-methylglucamide.

5. Process, according to claims 1 to 4, characterized in that the resuspended parasites are submitted to four cycles of freezing and thawing.

6. Process, according to claim 5, characterized in that the resultant lysate is centrifuged at 10,000g for 5 minutes at 4°C.

7. Test for the diagnosis of Chaga's disease, characterized in that 1- – 100 nanograms of A&T antigen and 40 – 100 nanograms of EpEx antigen, both

diluted in 50 – 100 mM carbonate-bicarbonate buffer, with a pH of 9.4 – 9.6, are added, separately, in white opaque wells of ELISA 96-well polystyrene test plates, and after 12 – 18 hours at 4 – 8°C, or 2 – 4 hours at 30 – 37°C, the plates are washed 3 – 5 times with 0.15 mM phosphate-buffered saline, with a pH of 7.3 – 7.5, containing 0.05 – 0,1% Tween 20 (PBS-T Buffer) and the free sites in the plates are blocked with 0.1 – 0.2% bovine serum albumin (BSA) in 50 – 100 mM sodium carbonate-bicarbonate buffer, with a pH of 9.4 – 9.6, for 12 – 24 hours at 4°C or, alternatively, for 1 – 4 hours at 35 – 37°C, the plates being washed 3 – 5 times with 300 ml of PBS-T buffer, and 50 – 100 ml of the sera, diluted 1:2,000 in PBS-T buffer containing 0.1 – 0.5% BSA (PBS-TB buffer), being added in each well; there following the incubation for 30 min. at 35 – 37°C, or 1 hour at 22 – 28°C, the plates being washed with PBS-T and the excess liquid being removed by inversion thereof on filter paper and thereafter, 50 – 100 ml of the biotinylated anti-human IgG conjugate, diluted 1:2,000 in PBS-TB buffer is added into each well and the plates being incubated for 5 – 30 min. at 35 – 37°C, or 1 hour at 22 – 28°C, being thereafter washed 3 – 5 times with 300 ml of PBS-T, the excess liquid being removed by inversion on filter paper and 50 – 100 ml of streptavidin-horseradish peroxidase conjugate diluted 1:1,000 – 1:2,000 in PBS-T buffer being added into each well, the plates being incubated for 30 min. at 35 – 37°C, or 1 hour at 22 – 28°C, and the plates being washed 3 – 5 times with 300 ml of PBS-T buffer, the excess liquid being removed by inversion on filter paper, followed by incubation of the plates for 5 – 30 min. at 22 – 28°C with the reagent Luminol®, diluted 1:10 – 1:20 in 50 – 100 mM carbonate-bicarbonate buffer, with a pH of 9.6 – 9.8 or, alternatively, diluted 1:5 – 1:10 in the same buffer containing 0.1 – 0.2% skimmed milk, the reaction being quantified using a luminometer for 96-well plate readings, and the results being expressed as Relative Luminescent Units (RLU).

8. Test, according to claim 7, characterized in that the cutoff value for each test plate, in the reaction of CL-ELISA with A&T or EpEx, is calculated using the following formula:

Plate cutoff value = (NC mean – B mean) x 10_

where:

NC mean = mean of the readings (RLU's) of negative control serum (at least 4 determinations per plate); B mean = mean of the readings (RLU's) of white (at least 4 determinations per plate), that is, of the reaction in the absence of serum.

9. Test, according to claim 7, characterized in that 10 nanograms of A&T antigen and 40 – 80 nanograms of EpEx antigen separately in plates are used.

10. Test, according to claims 7 to 9, characterized in that the ELISA plates are incubated for 12 hours at 4°C and washed 5 times with phosphate-buffered saline.

11. Test, according to claim 10, characterized in that the phosphate-buffered saline has a concentration of 0.15 mM and a pH of 7.5 with o.05% Tween 20.

12. Test, according to claims 7 to 11, characterized in that the free sites in the plates are blocked with 0.1% bovine serum albumin (BSA) in 50 mM sodium carbonate-bicarbonate buffer, with a pH of 9.6, for 12 hours at 4°C.

13. Test, according to claim 12, characterized in that the time is fo 2 hours and the temperature is 37°C.

14. Test, according to claims 7 to 13, characterized in that the plates are washed 5 times with 300 ml of the PBS-T buffer.

15. Test, according to claim 14, characterized in that, into each well 50 ml of the sera, containing 0.5% BSA (PBS-TB buffer), are added.

16. Test, according to claims 7 to 15, characterized in that it includes the incubation for 30 min. at 37°C.

17. Test, according to claims 7 to 16, characterized in that into each well 50 ml of the biotinylated anti-human IgG conjugate are added, and the plates are incubated for 30 min. at 37°C.

18. Test, according to claims 7 to 17, characterized in that 5 washing operations with 300 ml of PBS-T are carried out, the excess liquid being removed by inversion on filter paper and 50 ml of streptavidin-peroxidase conjugate diluted 1:1,000 in PBS-T buffer being added into each well and the plate being incubated for 5 –30 min. at 35 – 37°C, or 1 hour at 22 – 28°C. The plates are washed 3 – 5 times with 300 ml of PBS-T buffer, the excess liquid being removed by inversion on filter paper, and being thereafter incubated for 30 min. at the ambient temperature with the reagent Luminol®, diluted 1:20 in 50 mM carbonate-bicarbonate buffer with a pH of 9.6.

19. Test, according to claim 18, characterized in that the reagent Luminol® is diluted 1:5 in the same buffer containing 0.1 – 0.2% skimmed milk.

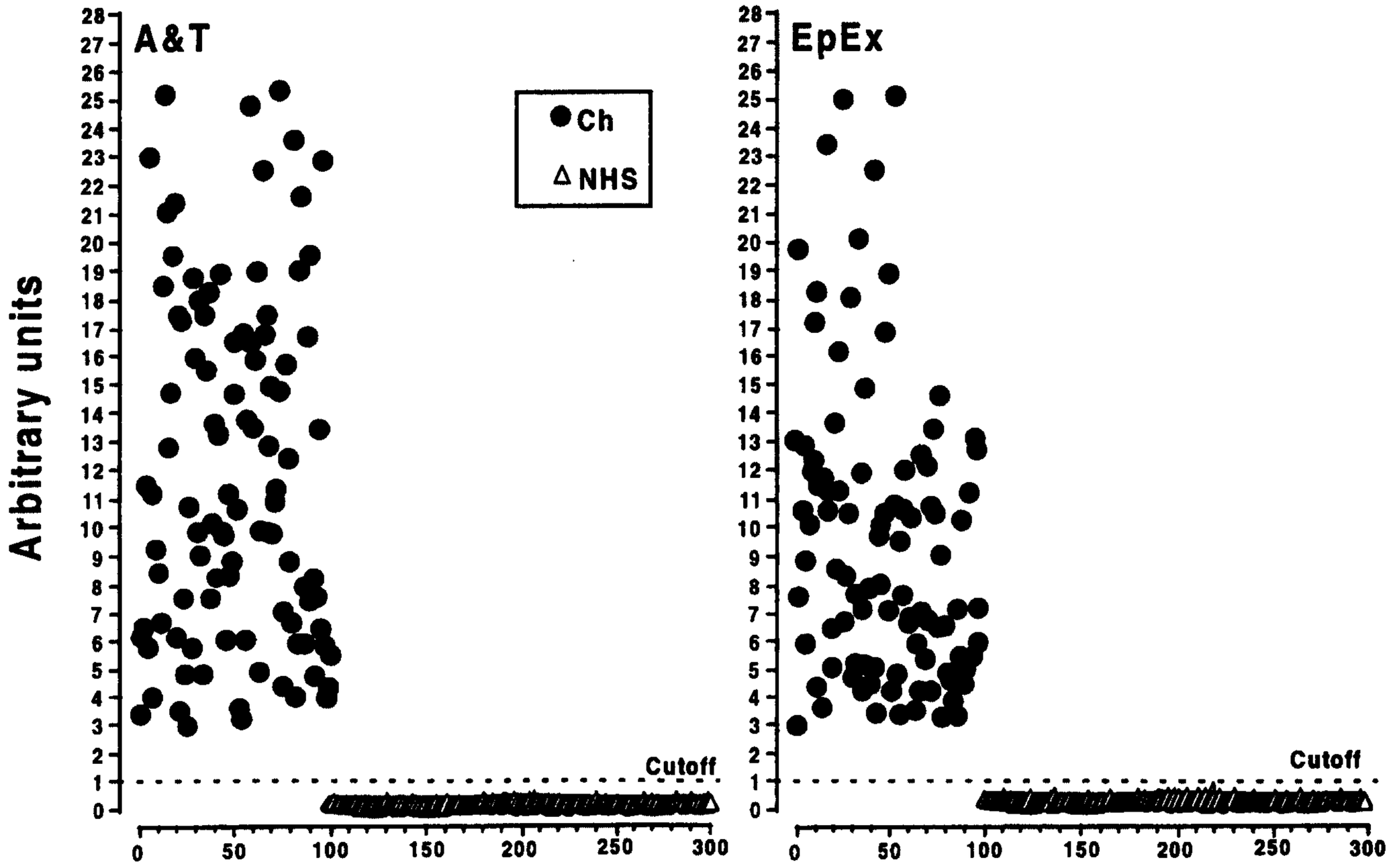

Fig. 1. Evaluation of the sensitivity of CL - ELISAs using A&T and EpEx antigens. Ch, chronic Chagasic sera (n=100); NHS, normal human sera (n=200).

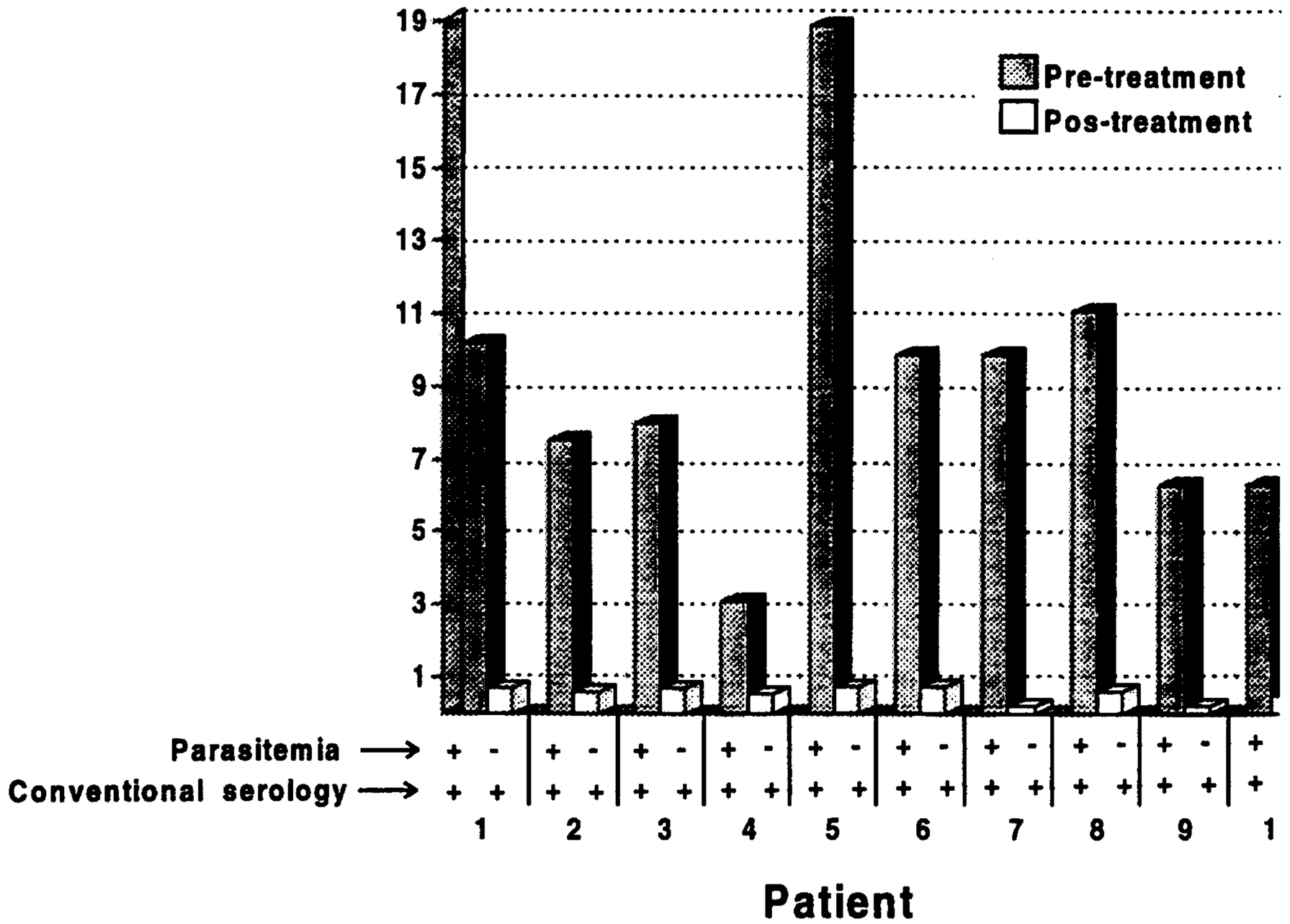

Fig. 2 - CL-ELISA using A&T antigen as a criterion of cure of treated Cha patients. Parasitemia was estimated by xenodiagnosis or hemocul Conventional serology: indiret hemagglutination, indirect immunofluorescence and Elisa.