

Antigenuria in Infants with Acute and Congenital Chagas' Disease

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Detection and partial characterization of *Trypanosoma cruzi* soluble antigens (SAg) in urine, as well as demonstration of parasite circulating antigens (CAg) in serum from pediatric patients with acute (10 patients) and congenital (10 patients) Chagas' disease, are reported. Classical techniques for parasite detection and antibody serology were also conducted in both groups. Samples collected before the onset of parasitocidal drug treatment were tested by an enzyme-linked immunosorbent assay for SAg and CAg demonstration. The control population consisted of 6 children with acute toxoplasmosis, 6 with cutaneous leishmaniasis, and 20 healthy individuals. Patients with acute cases were 100% positive for both SAg and CAg, whereas patients with congenital disease were 80% CAg positive and 100% SAg positive. Controls yielded negative results in all cases. Partial characterization of SAg from two patients with acute disease was performed by iodination, affinity chromatography, immunoprecipitation, and two-dimensional gel electrophoresis. Two different antigenic glycoproteins (80 kilodaltons, pI 6 to 6.5 and 55 kilodaltons, pI 6.5 to 7) were identified by these methods. Traditional serology and classical parasitologic tests failed, each in a different way, to provide an accurate diagnosis in the total of our patients. The enzyme-linked immunosorbent assay for SAg detection proved to be the most effective procedure for achieving early and precise proof of infection in acute and congenital cases of Chagas' disease.

Rapid diagnostic methods for acute and congenital cases of Chagas' disease are necessary because parasitocidal treatment is more effective the sooner it is started (14). Classical parasitologic diagnosis based on detection of the whole etiologic agent is limited by the delay in getting results. The development of specific, sensitive, and reliable techniques to demonstrate the presence of antibody-bound or free soluble antigens in different body fluids, such as serum or urine, is an important step toward improving diagnosis of this parasitic disease (28).

Circulating antigens (CAg) have been demonstrated in sera from *Trypanosoma cruzi*-infected animals (2, 12) and human patients (3, 22). We have reported detection of specific antigens and immune complexes by enzyme-linked immunosorbent assay (ELISA) in the sera of patients of different ages with acute and chronic Chagas' disease (17). The results of the few experiments so far performed to investigate the occurrence of *T. cruzi* soluble antigens (SAg) in urine seem contradictory. Araujo (2) was not able to detect SAg by ELISA in urine collected from infected mice. A report by Bongertz et al. (6) has demonstrated that urine from acutely infected mice and dogs contained antigen detectable by a double diffusion test. We have recently communicated the first demonstration of SAg in human urine samples (10). This work presents our results in the detection of *T. cruzi* antigens by ELISA in urine and serum specimens from patients with acute and congenital Chagas' disease. Partial characterization of SAg from two patients with acute disease is also shown.

MATERIALS AND METHODS

Techniques used for parasite detection. The microhematocrit (MH) procedure for parasite concentration (14), xeno-

diagnosis (XD) (9), and suckling mouse inoculation (SMI) (24) were performed simultaneously. (i) Six heparinized 50- μ l microhematocrit capillary tubes were filled with each infected blood sample and centrifuged at $3,000 \times g$ for 40 s, having been sealed at one end. Each tube was cut between the buffy coat and the erythrocyte pellet. The buffy coat was poured onto a slide and was microscopically examined for 20 min at $\times 400$ magnification. (ii) Ten to 40 (depending on the age of the patient) *Triatoma infestans* nymphs of the third stage were employed, and insects were dissected 30 days later to search for parasites in the digestive tract. (iii) Infected blood (50 μ l) was injected subcutaneously into each of 8 to 10 suckling mice, and parasitemia was evaluated in blood samples taken from the tails three times weekly for 40 days starting on day 15. MH was performed in all patients; XD and SMI were conducted in 13 of the 20 children.

Serologic tests. The direct agglutination test, before (DA) and after treatment of sera with 2-mercaptoethanol (DA-2ME) (26), and the indirect hemagglutination test (IHA) (8) (Laboratorios Polychaco, Buenos Aires, Argentina) were performed by standard procedures.

Anti-*T. cruzi* antisera for ELISA tests. One antiserum was obtained from two 2,500- to 3,000-g young male rabbits inoculated by the intramuscular route with 7.5×10^6 bloodstream RA strain parasites (15), purified from Rockland mice at the peak of parasitemia (7 days postinfection). The rabbits were bled 20 days after receiving parasites, and the sera were inactivated for 30 min at 56°C. The immunoglobulin fraction was separated by precipitation with ammonium sulfate (16) and purified by immunoabsorption with *T. cruzi* RA antigen (4, 17). The rabbit anti-*T. cruzi* immunoglobulins used to coat the ELISA plates were also absorbed with packed guinea pig erythrocytes (11) to rule out the possibility of antibodies in this preparation directed against any tissue antigens present in the studied samples and prevent false-positive results. The procedure was also performed with the

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TABLE 1. Antigenuria, antigenemia, parasitemia, and antibody serology in patients with acute and congenital Chagas' disease

Patient ^a	Age	Clinical signs	Evolution of illness (days) ^b	<i>T. cruzi</i> antigens		Parasitemia			Antibody titer		
				SAg	CAg	MH	XD	SMI	DA	DA-2ME	IHA
1A	6 mo	Chagoma, fever	23	+	+	+	+	+	1:4,000	1:64	1:256
2A	9 mo	Chagoma, fever	60	+	+	+	ND ^c	ND	1:32	1:32	1:16
3A	11 mo	Chagoma, generalized edema, hepatomegaly, myocardopathy	45	+	+	+	+	ND	1:16	1:16	<1:8
4A	1 yr	Chagoma	30	+	+	+	-	+	1:128	1:128	<1:8
5A	4 yr	Chagoma, fever, myocardopathy	20	+	+	+	ND	ND	<1:8	<1:8	<1:8
6A	5 yr	Chagoma, submaxillar adenopathy	25	+	+	+	ND	+	1:128	1:128	1:64
7A	5 yr	Chagoma, myocardopathy	75	+	+	+	+	+	1:256	1:32	<1:8
8A	6 yr	Chagoma, generalized edema	27	+	+	+	+	ND	1:64	1:64	1:16
9A	7 yr	Chagoma	17	+	+	+	+	+	1:16	1:16	<1:8
10A	10 yr	Chagoma, fever, submaxillar adenopathy	15	+	+	+	ND	+	1:2,000	1:64	<1:8
11C	17 days	Hepatomegaly, jaundice	17	+	+	+	ND	+	1:16	1:16	1:32
12C	24 days	Hepatitis	24	+	+	+	+	ND	<1:8	<1:8	<1:8
13C	2 mo	Asymptomatic	60	+	+	+	+	+	1:512	1:512	1:32
14C	4 mo	Hepatomegaly, splenomegaly	120	+	+	+	+	+	<1:8	<1:8	<1:8
15C	5 mo	Asymptomatic	150	+	-	+	+	+	<1:8	<1:8	<1:8
16C	6 mo	Asymptomatic	180	+	+	+	ND	ND	1:16	1:16	<1:8
17C	7 mo	Asymptomatic	210	+	+	-	+	-	1:64	1:64	1:32
18C	7 mo	Asymptomatic	210	+	-	-	+	-	1:64	1:64	<1:8
19C	8 mo	Hepatomegaly	240	+	+	+	ND	+	1:256	1:256	1:64
20C	8 mo	Hepatomegaly, splenomegaly, jaundice, myocardopathy	240	+	+	+	+	ND	1:64	1:64	1:8

^a A, Acute disease; C, congenital disease.

^b Time of infection considered since birth in congenital patients.

^c ND, Not done.

other antiserum, a pool of chronic chagasic patients' sera. Both antisera were filtered through a membrane (0.45- μ m pore size; Millipore Corp., Bedford, Mass.) and stored at -20°C until used. Specific antibodies against *T. cruzi* were demonstrated in these preparations by DA-2ME and IHA tests (titers, \geq 1:256).

ELISA for antigen detection in urine (ELISA-SAg) and serum (ELISA-CAg) samples. Microtiter polystyrene plates (Immulon 2; Dynatech Laboratories, Alexandria, Va.) with 96 flat-bottomed wells were coated with 100 μ l of rabbit anti-*T. cruzi* immunoglobulin (30 μ g of total protein per ml, as determined by the Lowry et al. method [21]) in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed three times in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). Wells were postcoated with 1% bovine serum albumin (Sigma) in PBS-Tween 20 and left at 37°C for 45 min. After washing, 100 μ l of serum diluted in PBS-Tween 20 or concentrated urine samples was added to each well and incubated for 2 h at 37°C. All samples were tested in duplicate. The plates were washed again, and 100 μ l of human anti-*T. cruzi* immunoglobulin diluted 1:100 in PBS-Tween 20 was added and incubated at 37°C for 60 min. Plates were washed three times and 100 μ l of conjugate (goat anti-human immunoglobulin G [IgG] labeled with alkaline phosphatase; Miles Laboratories, Inc., Elkhart Ind.), diluted 1:1,000 in PBS-Tween 20, was dispensed into each well. Optimal dilution of conjugate was determined as described previously (27). After 60 min at 37°C and thorough washing, 100 μ l of substrate (0.1% *p*-nitrophenyl phosphate [Sigma] in diethanolamine buffer with 0.5 mM magnesium chloride) was added, followed by 50 μ l of 3 M sodium hydroxide 30 min later. ELISA results were read with a Dynatech micro-ELISA reader (model MR 590) at a wavelength of 410 nm.

Positive or negative values were assigned by adding two standard errors to the mean absorbance of negative control samples. All readings were made against a blank row of wells receiving the above treatment, except that samples were replaced by PBS-Tween 20. Both *T. cruzi* (RA strain) and *Toxoplasma gondii*, prepared as described previously (17), and *Leishmania* spp. antigens (Behringwerke AG, Marburg, Federal Republic of Germany), diluted in urine or serum from uninfected persons to a final concentration of 34 μ g of protein per ml, were used as positive and cross-reaction controls, respectively. These samples received all of the above treatments for urine and serum specimens from infected patients.

Urine concentration procedure. Urine specimens were preserved with 0.2% sodium azide at 4°C until used. Whole trypanosomes were not observed in these urines. Samples were centrifuged at 3,000 \times *g* for 30 min and filtered through a membrane filter (0.45- μ m pore size) before precipitation with 3 volumes of cold ethanol for 48 h at -20°C. The concentrated pellet was obtained after centrifugation at 9,800 \times *g* for 30 min at 4°C and suspended in 0.5 ml of PBS, pH 7.2 (18). Specimens concentrated 10-fold with a Minicon B-15 concentrator (Amicon Corp., Texington, Mass.) were also tested.

Human patients. Urine and serum samples were obtained from 10 patients (aged 6 months to 10 years) with acute *T. cruzi* infections and 10 patients (aged 17 days to 8 months) with congenital *T. cruzi* infections. The patients with acute infection who lived in central and northeastern Argentina, a well-known endemic area, presented inoculation chagoma, a portal-of-entry sign characterized by painless unilateral edema of the eyelids. The congenitally infected patients were born in a nonendemic area from mothers serologically positive for Chagas' disease, and they had not received any

blood transfusion. Clinical details are summarized in Table 1. Both groups showed parasitemia, detectable by at least one of the classical tests for parasite demonstration (MH, SMI, XD). Samples were collected before the beginning of parasitocidal drug treatment (Nifurtimox, 15 mg/kg of body weight per day for 90 days). All sera were filtered through a membrane (0.45- μ m pore size) and then stored at -20°C until used. The control population consisted of 12 children, 6 with acute toxoplasmosis and 6 with cutaneous leishmaniasis, and 20 healthy individuals, none of them with detectable antibodies against *T. cruzi* (titers, $<1:8$ by DA-2ME and IHA tests). Urine and serum specimens were collected from this group and used as controls for the ELISAs.

Iodination of concentrated urines. The pellets obtained by the urine concentration procedure were lyophilized and then suspended in 1 ml of buffer containing 140 mM NaCl, 10 mM Tris hydrochloride (pH 8.6), 1 mM phenylmethylsulfonyl fluoride, and 1 mM *N*- α -*p*-tosyl-L-lysyl chloromethyl ketone. The samples were incubated in 1 ml of buffer containing 300 μCi of Na^{131}I per ml in glass tubes precoated with 20 μg of Iodo-gen (13). After 10 min at 4°C , the suspensions were exhaustively dialyzed against 10 mM Tris hydrochloride (pH 8.6).

Lectin affinity chromatography. Radioiodinated materials were treated with 2% Nonidet P-40 and applied directly to a lectin-Sepharose column (volume, 0.6 ml; height, 5 cm). Concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The column was equilibrated in 140 mM NaCl–10 mM Tris hydrochloride (pH 8.6) containing 2% Nonidet P-40 before adsorption of the sample. Nonadsorbed material was eluted with the same buffer at a constant flux of 0.2 ml/min. Fractions of 1 ml were collected, and the radioactivity was measured directly. Bound material was eluted with the same buffer containing a mixture of 100 mM each α -methyl D-mannoside and α -methyl D-glucoside. The carbohydrate-eluted material was precipitated by the addition of 3 volumes of cold ethanol at -20°C for 48 h.

Immunoprecipitation. The precipitated material obtained after lectin affinity chromatography was incubated overnight with 50 μl of rabbit antiserum to *T. cruzi*, trypomastigote form specific (1), and the antigen-antibody complexes were left for 30 min at room temperature with 70 μl of a 10% suspension of heat-killed and Formalin-fixed *Staphylococcus aureus* Cowan I cells (19). The immune complexes were washed twice as recommended previously (19) and finally with a buffer containing 0.05% (vol/vol) Nonidet P-40, 0.1% (wt/vol) sodium dodecyl sulfate, 300 mM NaCl, and 10 mM Tris hydrochloride (pH 8.6). The precipitates were suspended in 70 μl of two-dimensional electrophoresis sample buffer (23) and centrifuged at $10,000 \times g$ for 30 min, and the supernatants were subjected to isoelectric focusing.

Two-dimensional polyacrylamide gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was performed under equilibrium conditions as described by O'Farrell (23). Ampholines in three different pH ranges (1% pH 3.5 to 10.0; 0.7% pH 4.0 to 6.0; 0.3% pH 9.0 to 11.0) were combined to obtain a broad pH gradient. After isoelectric focusing (16 h at 400 V) the pH gradient was measured directly on the first-dimension gel by means of a surface electrode. As commented by O'Farrell (23), direct measurement of pH on urea gels may not give precise values. Thus, all references to isoelectric pH values found in this work are only approximate and have been used for descriptive purposes. The second-dimension slab gels were prepared as linear 7 to 14% gradients. The following molecular mass markers were used: trypsinogen (24,000), ovalbumin

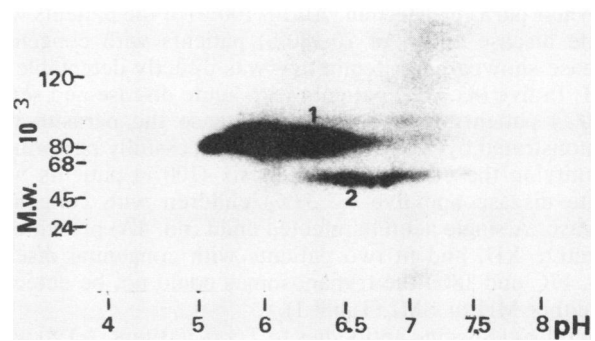


FIG. 1. Autoradiograph of ^{131}I -labeled *T. cruzi* antigens eliminated in the urine of one patient with acute disease; the antigens were isolated by affinity chromatography and immunoprecipitation and analyzed by two-dimensional gel electrophoresis. M.w., Molecular weight.

(45,000), bovine serum albumin (68,000), human transferrin (80,000), and β -galactosidase (120,000). Gels were dried and exposed to Kodak X-RP5 films for periods from 7 to 15 days.

RESULTS

Results of ELISA in urine and serum samples. Results of detection of *T. cruzi* SAg in urine specimens and CAg in sera from patients with acute and congenital Chagas' disease are shown in Table 1. All acute cases were positive by both ELISA-SAG and -CAg. Out of 10 congenital chagasic patients, 8 (80%) were ELISA-Cag positive and 10 (100%) were ELISA-SAG positive. These results were obtained with urines concentrated by the ethanol precipitation technique, whereas only 60% of acute cases and 40% of congenital cases were ELISA-SAG positive with a Minicon concentrator (data not shown). Antigens could not be detected in untreated urines. *T. cruzi* standard antigen, diluted in urine or serum from nonchagasic individuals, yielded net absorbance values exceeding the cutoff level of the assay. Samples from uninfected people, patients with acute toxoplasmosis and cutaneous leishmaniasis, and urine or serum specimens containing *T. gondii* and *Leishmania* spp. antigens gave negative results in all cases.

Antigen characterization. Partial characterization of parasite antigens eliminated in urine from two acutely infected patients (no. 3A and 6A), who presented the biggest pellets, was performed. Affinity chromatography on concanavalin A-Sepharose demonstrated the glycoprotein nature of these *T. cruzi* antigens. The analysis of carbohydrate-eluted material showed two peaks of radioactivity for each sample. When both peaks were immunoprecipitated with specific antiserum to *T. cruzi*, only the second one proved to contain antigenic glycoproteins as revealed by polyacrylamide gel electrophoresis. Two glycoproteins with molecular masses of approximately 80 and 55 kilodaltons, respectively, were identified by this method. No antigens could be recognized in the first peak of nonspecific radioactivity. Urines from nonchagasic patients showed only this first peak. Negative results were also obtained by immunoprecipitation with normal rabbit serum, always employed as the control serum. Finally, two-dimensional analysis (Fig. 1) revealed a few spots with a small range of isoelectric points. One glycoprotein (80 kilodaltons) was identified between pH 6.0 and 6.5 (glycoprotein 1). Another glycoprotein (55 kilodaltons, pI 6.5 to 7.0) exhibited satellite spots with the same molecular mass, suggesting that charge heterogeneity might be occurring (glycoprotein 2).

Whole parasite detection. All 10 (100%) of the patients with acute disease and 8 of 10 (80%) patients with congenital disease showed parasitemia that was directly detectable by MH. In five (83.33%) patients with acute disease and seven (100%) patients with congenital disease the parasite was demonstrated by XD, and SMI was successfully performed, identifying the etiologic agent in six (100%) patients with acute disease and five (83.33%) children with congenital disease. A single acutely infected child (no. 4A) presented a negative XD, and in two patients with congenital disease (no. 17C and 18C) the trypanosomes could not be detected by either MH or SMI (Table 1).

Serology. Specific antibodies to *T. cruzi* (titers, $\geq 1:8$) were detected, at least by one technique, in 16 (80%) of our 20 patients (Table 1). In three acutely infected patients (no. 1A, 7A, and 10A) a difference between titers obtained by DA and DA-2ME was observed, suggesting the occurrence of acute infection. No significant differences were found in the remaining patients, indicating that IgM antibody response could not be demonstrated. The IHA test showed only 45% positive whereas 80% of the studied children were serologically reactive by the DA test.

DISCUSSION

In the present work, the ELISA-SAg proved to be the most effective procedure in achieving an early and precise diagnosis in all of our patients with acute and congenital Chagas' disease.

The results obtained in the search for the parasite and in detection of specific antibodies to *T. cruzi* show that Chagas' disease diagnosis still offers difficulties. In our series, for different reasons neither the traditional serology nor the methods for parasite demonstration were capable of providing an accurate diagnosis of all of the patients studied. Serologic tests were helpful for diagnosis of recently acquired infection in only three of our patients with acute cases, who showed differences in titers (threefold or greater) between DA and DA-2ME, and in five patients with congenital disease, who had detectable antibodies after the first 6 months of life. Moreover, specific antibodies could not be determined by either DA or IHA tests in one patient with acute disease and three patients with congenital disease at the time of sampling.

XD, considered the most sensitive method, failed to demonstrate the presence of circulating parasites (visualized by both MH and SMI) in one patient with acute disease. Unfortunately, XD could not be repeated under the original conditions, because specific treatment was immediately started. However, in two patients with congenital disease who had negative results with MH and SMI, both excellent methods as regards sensitivity, diagnosis was only confirmed by XD. This result could be attributed to low parasitemia levels as a consequence of prolonged infection. Although parasitemia was detectable by at least one of the three parasitologic tests performed in all infected children, XD and SMI are expensive and not always practical procedures and are also limited by the delay in getting the final result. The very simple and rapid MH technique showed a slightly lower sensitivity than our ELISA-SAg assay. MH is a direct micromethod suitable for routine diagnosis in cases of transplacental or recently acquired *T. cruzi* infection. However, despite its advantages, it is troublesome to perform simultaneously on a large series of patients because the average microscope observation time is 20 min per sample.

In highly endemic areas, where many suspected acute chagasic persons must be tested in the laboratory, the ELISA-SAg has proved more adequate for large-scale assays.

Demonstration of CAg in sera from acutely infected children yielded 100% positivity by ELISA. These results are similar to those obtained by Kahn et al. (17). Patients with congenital disease were 80% ELISA-CAg positive. The two children in whom we failed to confirm antigenemia presented urinary excretion of *T. cruzi* antigens. Both patients were asymptomatic, and samples were collected at 150 and 210 days of life, respectively. ELISA-SAg showed higher sensitivity, since the urine concentration procedure increases the chances of demonstrating the presence of parasite antigens undetectable by ELISA-CAg in diluted serum.

The detection of *T. cruzi* SAg in urine from infected patients offers certain new advantages. First, it promises to be of diagnostic value; all patients with acute and congenitally acquired disease were positive by the ELISA for antigens in urine before the onset of treatment with parasitocidal drugs. At the same time, all of them showed parasitemia detectable by at least one of the parasitologic tests already described. Second, samples are obtained in a simple and painless way, without the need to take blood samples. Venipuncture under field conditions requires skilled technicians and expensive materials and poses a significant risk to the patient (28). Another characteristic of the assay is its specificity; no cross-reactivity was found with the controls employed.

During the course of specific treatment, whenever parasitemia became negative, antigens could not be detected in the urine in most of these patients (data not shown). This correlation suggests that SAg could be helpful in evaluating the susceptibility of the parasite to drug treatment (10).

During the setting up of our ELISA test we found that it was necessary to concentrate urine samples to improve sensitivity, since antigens could not be demonstrated in untreated urine specimens. The use of a Minicon concentrator was only partly satisfactory; the best results were obtained with the ethanol precipitation technique (10-fold concentration), suggesting that the parasite antigens eliminated in urine were mainly glycoproteins (25). This was confirmed by lectin affinity chromatography and two-dimensional polyacrylamide gel electrophoresis under equilibrium conditions. Glycoprotein 1 (80 kilodaltons, pI 6 to 6.5) resembles the surface glycoprotein described previously by Andrews et al. (1) and by Lanar and Manning (20). Glycoprotein 2 (55 kilodaltons, pI 6.5 to 7) may correspond to tubulin or another internal component of trypomastigote or amastigote forms. Satellite spots were observed for this glycoprotein in our system. The possible modification in vivo responsible for the charge heterogeneity could be sialylation and phosphorylation (5), as described for African trypanosomiasis. Both glycoproteins contain mannose or glucose or both, since they are retained by concanavalin A-Sepharose columns.

We believe that ELISA for *T. cruzi* SAg detection and the MH procedure represent a powerful combination of tests to confirm early diagnosis of Chagas' disease and are particularly useful in acute and congenital cases where conflictive serology is frequently observed. Further studies are required to ensure a better identification and understanding of these new infection markers. The production of monoclonal antibodies directed to fully characterized *T. cruzi* antigens eliminated in urine may improve assay sensitivity and allow a more accurate diagnosis of this protozoan infection to be achieved.

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LITERATURE CITED

- Andrews, N. W., A. M. Katzin, and W. Colli. 1984. Mapping of surface glycoproteins of *Trypanosoma cruzi* by two-dimensional electrophoresis. A correlation with the cell invasion capacity. *Eur. J. Biochem.* **140**:599-604.
- Araujo, F. G. 1982. Detection of circulating antigens of *Trypanosoma cruzi* by enzyme immunoassay. *Ann. Trop. Med. Parasitol.* **76**:25-36.
- Araujo, F. G., E. Chiari, and J. C. P. Dias. 1981. Demonstration of *Trypanosoma cruzi* antigen in serum from patients with Chagas' disease. *Lancet* **i**:246-249.
- Avrameas, S., and T. Ternynck. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry* **6**:53-66.
- Baltz, T., C. Giroud, D. Baltz, G. Duvillier, P. Degand, J. Demaille, and R. Pautrizel. 1982. The variable surface glycoproteins of *Trypanosoma equiperdum* are phosphorylated. *EMBO J.* **1**:1393-1398.
- Bongertz, V., K. Hungerer, and B. Galvao-Castro. 1981. *Trypanosoma cruzi*: circulating antigens. *Mem. Inst. Oswaldo Cruz* **76**(Suppl. 1):71-82.
- Camargo, M. E., M. C. S. Guimarães, B. A. Peres, and S. Hoshino-Shimizu. 1976. Antigenic fractionation and development of new antigens, p. 212-215. *In* New approaches in American trypanosomiasis research. Scientific Publication no. 318. Pan American Health Organization, Washington, D.C.
- Cerisola, J., M. Fatała Chaben, and J. Lazari. 1967. Test de hemoaglutinación para el diagnóstico de la enfermedad de Chagas. *Prensa Méd. Argent.* **49**:1761-1765.
- Cerisola, J., R. Rohwedder, G. E. del Prado, M. Alvarez, and G. J. W. de Martini. 1974. El xenodiagnóstico, normalización, utilidad. *Publicación del Ministerio de Bienestar Social, Secretaría de Estado de Salud Pública, Buenos Aires.*
- Corral, R., H. Freilij, A. Montemayor, and S. Grinstein. 1984. *Trypanosoma cruzi* antigens in urine from patients with Chagas' disease. *IRCS (Int. Res. Commun. Syst.) Med. Sci. Libr. Compend.* **12**:1034-1035.
- Cossio, P. M., C. Diez, A. Szarfman, E. Kreutzer, B. Candiolo, and R. Arana. 1974. Demonstration of a serum gamma globulin factor which reacts with endocardium and vascular structures. *Circulation* **49**:13-21.
- De Siqueira, A. F., F. Ferrioli Filho, and R. Domingues Ribeiro. 1979. Aspectos imunitários iniciais observados em ratos infectados por *Trypanosoma cruzi*. II. A circulação de antígenos solúveis e as modificações do complemento sérico dos animais em dias sucessivos da infecção. *Rev. Bras. Pesqui. Méd. Biol.* **12**(Suppl. 1):75-79.
- Fraker, F. J., and J. C. Speck. 1978. Protein and cell membrane indications with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80**:849-857.
- Freilij, H., L. Muller, and S. M. Gonzalez Cappa. 1983. Direct micromethod for diagnosis of acute and congenital Chagas' disease. *J. Clin. Microbiol.* **18**:327-330.
- Gonzalez Cappa, S. M., A. T. Bijovsky, H. Freilij, L. Muller, and A. M. Katzin. 1981. Aislamiento de una cepa de *Trypanosoma cruzi* a predominio de formas delgadas, en Argentina. *Medicina (Buenos Aires)* **41**:119-120.
- Herbert, G. A., P. L. Pelham, and B. Pittman. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse and goat antisera. *Appl. Microbiol.* **25**:26-36.
- Kahn, T., R. Corral, H. Freilij, and S. Grinstein. 1983. Detection of circulating immune complexes, antigens and antibodies by enzyme-linked immunosorbent assay in human *T. cruzi* infection. *IRCS (Int. Res. Commun. Syst.) Med. Sci. Libr. Compend.* **11**:670-671.
- Katzin, A., and W. Colli. 1983. Lectin receptors in *Trypanosoma cruzi*. An N-acetyl-D-glucosamine-containing surface glycoprotein specific for the trypomastigote stage. *Biochim. Biophys. Acta* **727**:403-411.
- Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617-1624.
- Lanar, D., and J. Manning. 1984. Major surface proteins and antigens on the different in vivo and in vitro forms of *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **11**:119-131.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Marcipar, A., S. Barnes, E. Lentwojt, and G. Brown. 1982. Immunoenzymatic determination of antibody-bound soluble antigens of *Trypanosoma cruzi*. *Appl. Biochem. Biotechnol.* **7**:459-462.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
- Phillips, N. R. 1960. Experimental studies on the quantitative transmission of *Trypanosoma cruzi*: considerations regarding the standardization of materials. *Ann. Trop. Med. Parasitol.* **54**:60-70.
- Spiro, R. G. 1966. Characterization of carbohydrate units of glycoproteins. *Methods Enzymol.* **8**:29-32.
- Vattuone, N. H., and J. F. Yanovsky. 1971. *Trypanosoma cruzi*: agglutination activity of enzyme-treated epimastigotes. *Exp. Parasitol.* **30**:359-363.
- Voller, A., D. Bidwell, and A. Bartlett. 1980. Enzyme-linked immunosorbent assay, p. 359-371. *In* N. R. Rose and H. Friedman (ed.), *Manual of clinical immunology*, 2nd ed. American Society for Microbiology, Washington, D.C.
- Voller, A., and D. De Savigny. 1981. Diagnostic serology of tropical parasitic diseases. *J. Immunol. Methods* **46**:1-29.