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HEMAGGLUTINATION TEST FOR CHAGAS' DISEASE WITH CHROMIUM CHLORIDE, FORMALIN-TREATED ERYTHOCYTES, SENSITIZED WITH *TRYPANOSOMA CRUZI* EXTRACTS

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SUMMARY

Fixing *Trypanosoma cruzi* extracts to formalin-treated human erythrocytes through the action of chromium chloride resulted in sensititized cells which could be kept in the refrigerator, for immediate use, for at least a few months, with no diminished antigenic activity. The hemagglutination test, most conveniently performed in microplates, seems practical for serologic surveys and large routine work and can be done with finger-tip blood samples. Different extraction procedures were employed and better results were obtained with ether-treated extracts resulting from the lysis of parasites with a 0.15% sodium desoxycholate water solution. Optimum sensitizing protein concentration of antigenic extracts ranged from about 10 to 50 micrograms per milliliter.

Although cross-reactivity was the rule for visceral and muco-cutaneous leishmaniasis, initial results here reported, for 190 cases of American trypanosomiasis and 200 other patients, indicate sensitivity and specificity of the test as satisfactory, which justifies a wider study of this technique.

INTRODUCTION

The widespread incidence of Chagas' disease and the many millions of people exposed to the risk of *T. cruzi* infection, in Latin America, indicate the importance of the development of practical serologic techniques for its diagnosis. Such tests must be as simple as possible to be used in surveys, as well as in blood banks and clinical laboratories.

The hemagglutination technique has been successfully applied to the diagnosis of Chagas' disease (CERISOLA et al. [3]; KNIERIM & SAAVEDRA [8]). As the immunofluorescence test, the hemagglutination test is economical in the amounts of reagents and allows the use of finger-tip blood samples, collected on filter paper (HOSHINO et al. [7]). It has the further advantage of prescinding elaborated devices, such as the fluorescence microscope.

However, fresh erythrocytes are not practical for routine hemagglutination tests, since new batches of cells must be daily sensitized. In this way, we have been employing formalin treated human erythrocytes, to which *T. cruzi* antigens were fixed through the action of chromium chloride, as referred by GOLD & FUDENBERG [5]. Such sensitized cells can be kept at 4ºC for many weeks with no diminished antigenic activity. In this paper we present the first results so obtained.

MATERIAL AND METHODS

Cultures of Trypanosoma cruzi — The Y strain (SILVA & NUSSENZWEIG [10]) of *T. cruzi* was cultivated in a liquid medium described

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by YAEGER, as referred by FERNANDES & LASTELLANI [4], with a yield of about 6 g of wet sediment per liter of medium.

Cultures were held at 28°C for 7-8 days, and almost only parasites in the epimastigote stage (HOARE & WALLACE [6]) were obtained. After centrifuging the cultures, the sediment was washed three times in large volumes of PBS (0.15 M NaCl 0.01 M phosphates; pH 7.2). When not used in the same day, sediments were vacuum dried at-20°C and kept at this temperature.

Serums — Were obtained from patients with clinical and serological evidences of Chagas' disease, many of whom showed positive parasitological tests, from patients with other diseases, parasitic or not, and from apparently normal individuals. Such serums had been kept for varying periods at -20°C and were not decomplemented by heating before tests. For use, serums were diluted at 1:40 in 0.15 M NaCl containing 2% normal rabbit serum, which had been previously absorbed with human group O, Rh positive erythrocytes. For titrations, twofold serum dilutions were used.

A positive standard serum was prepared by pooling serum samples from parasitologically proven cases of American trypanosomiasis, distributing in ampoules and freeze-drying.

Trypanosoma cruzi antigens — Different techniques were employed to prepare *T. cruzi* antigenic extracts to sensitize erythrocytes. Vacuum-dried parasites were ground with cold absolute ethanol and with cold ethyl ether. After drying by suction, the powder was suspended in 0.15 M NaCl or in 0.15% sodium desoxycholate in distilled water, and extracted overnight at 4°C, under slow rotation with glass-beads. The mixtures were centrifuged and the supernatants kept at -20°C. Sediments of saline extractions were suspended in smaller volumes of saline solution and extracted again in the same way.

Wet sediments of fresh cultures were washed in saline solution and suspended in about one-tenth of the original culture volume of distilled water, or of 0.15% water solution of sodium desoxycholate. The mixture was kept under slow rotation, with glass-beads, overnight, at 4°C, and then centrifuged at about 1,000 g, in the cold.

When desoxycholate was employed in the extracting processes, supernatants were dialysed against 0.15 M NaCl for about 36 hours at 4°C. Supernatants were kept at -20°C. Before use, they were centrifuged to remove eventual precipitates and protein concentration determined by the technique of LOWRY et al. [9]. For sensitization, extracts were diluted in saline solution.

Hemagglutination test — Human erythrocytes, group O, Rh negative were formolized as described by BUTLER [1], and stored at 4°C as a 10% suspension in 0.15 M NaCl. For sensitization, an aliquot of this suspension was centrifuged and the sedimented cells resuspended at 2.5% in the working saline dilution of the *T. cruzi* extract. A 0.8% solution of chromium chloride ($CrCl_3.6H_2O$) in distilled water was them added, 0.4 ml of solution for every milliliter of cell suspension, and the mixture kept for 7 to 10 minutes at room temperature. After this period, the mixture was diluted to about twenty times its volume with saline solution and centrifuged for a few minutes to sediment cells. These were washed twice, and suspendend at 2.7%, in 0.15M NaCl containing merthiolate at 10^{-4} and kept at 4°C. For use, one drop of normal rabbit serum was added to every 50 drops of this suspension.

The tests were perfomed in lucite plates containing V-shaped wells of about 0.15 ml capacity(*), or in similar disposable plastic trays(**), with four drops (0.1 ml) of serum dilutions, plus one drop (0.025 ml) of sensitized cells suspension. Blanks were prepared for serums (1:40 dilution plus non-sensitized cells), and for antigen (diluent plus sensitized cells). Readings were taken after 4 or 5 hours or after keeping plates overnight at room temperature. Sedimentation patterns were evaluated as previously indicated for similar tests (HOSHINO et al. [7]), negative results corresponding to a compact button of deposited cells. In strongly positive tests, an irregular carpet of sedimented cells

(*) Microtitrator Takatsy, Labor, Budapest, Hungary
(**) Linbro Chemical Co, or Cooke Engineering Co, USA

formed, which was seen in weakly positive tests as a narrow ring around a smooth mat deposit of erythrocytes. Titers of serums were taken as the highest dilution giving an unquestionable although weak agglutination of cells.

RESULTS

Optimal sensitization of cells, as translated by maximum titers for the positive standard serum, were obtained only with protein concentrations of several hundred micrograms per milliliter of solution when protein-rich saline extracts were employed. However, for second saline extracts and for desoxycholate or distilled water extracts, much smaller protein concentrations were necessary as indicated in Table I.

For an efficient sensitization, whenever parasites had not been previously delipidized, extracts had to be treated with ethyl ether. About one half volume of cold ether (-20°C) was added to the cold extract (4°C) and the mixture vigorously shaken for a few seconds and quickly centrifuged at 4°C. The water layer was collected, the protein concentration determined and dilutions for use prepared according to this concentration.

Sensitized cells kept at 4°C for varying periods have shown no diminished antigenic activity for as long as three months, the longest period of observation.

TABLE I

Titers of a Chagas' standard serum for different *T. cruzi* antigenic extracts (Hemagglutination test with chromium chloride, formalin - treated human erythrocytes)

EXTRACTS (*)	Protein concentration (μg/l)		Serum titers
	Total	Minimum, optimum sensitizing	
A = Vacuum — Dried parasites			
1 — Delipidized, 1st saline extraction	3,760	350	5,120
	3,040	460	5,120
	6,080	750	5,120
	2,600	780	2,560
	7,200	650	5,120
	1,360	500	5,120
2 — Second saline extraction	848	14	5,120
	1,184	18	2,560
3 — Extracted in 0.15% Na desoxycholate	5,600	30	5,120
	1,040	52	5,120
	704	10	2,560
B = Culture wet sediment			
1 — Lysed in distilled water	1,440	20	5,120
	1,000	40	5,120
	544	38	2,560
2 — Lysed in 0.15% Na desoxycholate	3,100	52	2,560
	1,320	52	2,560
	1,920	20	2,560
	448	10	2,560

(*) Treated with cold ethyl-ether

TABLE II

Results of hemagglutination and immunofluorescence tests in 152 cases of American trypanosomiasis

Immunofluorescence	Hemagglutination titers								
	< 40	40	80	160	320	640	1,280	2,560	5,120
< 20									
20		1							
40				1	1			1	
80				1		3			
160			3	4	2	6	5	2	
320			2	3	8	19	16	19	2
640					1	7	12	13	2
1,280						3	4	6	5

TABLE III

Results of the hemagglutination test for Chagas' disease

CASES	no.	Hemagglutination test	
		Positive	Negative
A — Individuals without hemoflagellate infections:			
Positive syphilis serology	16	0	16
High anti toxoplasma titers	20	0	20
Active toxoplasmosis (wtih IgM antibodies)	8	1	7
High anti-treptolysin O titers	23	0	23
Active rheumatic fever	2	0	2
Rheumatic arthritis	5	1	4
Hashimoto's thyroiditis	3	0	3
Mononucleosis	2	1	1
Histoplasmosis	1	0	1
South American blastomycosis	1	0	1
Healthy individuals	119	0	119
TOTAL	200	3 (1.5%)	197 (98.5%)
B — Hemoflagellate infections:			
Kala-Azar	6	6	0
Muco-cutaneous leishmaniasis	6	6	0
American trypanosomiasis	190	190	0
TOTAL	202	202 (100%)	0

In serum from 100 cases of Chagas' disease, positive hemagglutination tests were seen in all. Hemagglutination titers were in general higher than immunofluorescence titers, as indicated by comparative titrations performed in 152 serums (Table II).

In patients presenting no *T. cruzi* infection and in individuals considered as healthy, only a small percentage of positive tests (1.5%) was observed (Table III), plus several doubtful results which were considered as negative. From the three positive cases, one had mononucleosis, showing a high heterophil antibody titer, one had active toxoplasmosis, with anti-toxoplasma IgM antibodies, and one had rheumatoid arthritis with a positive latex test. However, in 12 cases of leishmaniasis, both visceral and muco-cutaneous, results were positive, with titers as high as those found in cases of American trypanosomiasis.

DISCUSSION

The effective fixation of *T. cruzi* antigens on human formalin-treated erythrocytes, through the action of chromium chloride, as here demonstrated, contributes to make the hemagglutination test for Chagas' disease a practical technique for routine purposes. The use of chromium chloride as a binding agent is a more simple and more reproducible process than when tannic acid is employed. The erythrocyte sensitizing technique is thus reduced to adding the chromium chloride solution to cells already suspended in the antigenic solution. No buffers are needed either for the sensitizing stage or for the dilution of serum, and the binding agent is an analytical grade chemical of constant activity for different batches. It can be kept for several weeks at 4°C as an 8% stock solution to be diluted for use (0.8%).

Sensitized cells have maintained an undiminished antigenic activity for many weeks, even for months, when kept in the refrigerator, ready for immediate use.

Among the different extracts employed best results were obtained with those produced through the lysis of *T. cruzi* in 0.15% sodium desoxycholate water solution. These gave clear-cut differences between reactive and non-reactive serums, with negative tests represented by a neat button of packed cells. For such extracts, antigenic protein concentration necessary for optimum sensitization of cells, as translated by maximum serum titers, were found to vary between narrow limits, from about 10 to 50 micrograms per milliliter, when much larger amounts had to be used for the protein-richer saline extracts. This suggests the presence of non-sensitizing protein components in these extracts. However, second saline extractions of *T. cruzi* sediments yielded antigens similar to those obtained through lysis of the parasites in distilled water or in desoxycholate water solutions.

Ether extraction of antigenic solutions resulted in much better sensitizing preparations than with total extracts. A large amount of material was so removed, represented by an opalescent layer between the ether and water phases and traduced by a fall in the protein concentration of the solution.

Other features of the technique here described make it practical for routine work. Since the sensitized cells are human O, Rh negative erythrocytes, samples for testing have not to be previously absorbed, as it is the case for sheep cells.

The perfomance of the test in microplates results in economy of reagents and glassware, a fundamental aspect for large scale work. Also, it permits the use of finger-tip blood samples, collected on filter paper, a most convenient detail for surveys, since it so avoids the complexities related to collection of venous blood, separation and conservation of serum. Since stable at room temperature for a few weeks (SOUZA & CAMARGO [11]) blood samples are more easily kept than serum because refrigerating conditions can be dismissed and common mailing facilities are satisfactory for sending samples to central laboratories.

Our initial results here reported were sufficiently sensitive and specific.

Cross-reactions, however, as already observed in the immunofluorescence test (CAMARGO & REBONATO [2]) were the rule for leishmaniasis, either visceral or muco-cutaneous.

CAMARGO, M. E.; HOSHINO, S.; CORRÊA, N. S. & PERES, B. A. — Hemagglutination test for Chagas' disease with chromium chloride, formalin-treated erythrocytes, sensitized with *Trypanosoma cruzi* extracts. *Rev. Inst. Med. trop. São Paulo* **13**:45-50, 1971.

We feel such results justify a wider study on the application of this test in the routine serological diagnosis of American trypanosomiasis.

RESUMO

Reação de hemaglutinação para a doença de Chagas, com hemácias formolizadas e sensibilizadas com extratos do T. cruzi *pelo cloreto de cromo*

O cloreto de cromo mostrou-se eficiente para a fixação de antígenos de *T. cruzi* a hemácias humanas formolizadas. As suspensões de hemácias sensibilizadas vêm se mantendo antigênicamente ativas, por meses, a 4ºC. De vários extratos antigênicos experimentados, aquêles resultantes da lise de formas de cultura do *T. cruzi* por solução aquosa de desoxicolato de sódio a 0,15% deram os melhores resultados, depois de tratados pelo éter etílico. As concentrações ótimas para a sensibilização variaram entre 10 e 50 microgramas de proteínas por mililitro de solução antigênica. A sensibilidade e a especificidade da reação parecem satisfatórias, mas nos soros de pacientes com leishmanioses, as reações foram positivas. Feita em microplacas, a reação é econômica no consumo de reativos e se presta a inquéritos populacionais, podendo ser realizada com amostras de sangue colhidas por picada digital, em papel de filtro.

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