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HEMAGGLUTINATION WITH PRESERVED, SENSITIZED CELLS, A PRACTICAL TEST FOR ROUTINE SEROLOGIC DIAGNOSIS OF AMERICAN TRYPANOSOMIASIS

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SUMMARY

Aldehyde-preserved human erythrocytes were sensitized with *T. cruzi* extracts and then treated with formalin and freeze-dried. The hemagglutination test with such a reagent could be standardized more easily and was more practical for routine purposes than when fresh cells were employed. A comparative study with 1,430 serum samples showed very similar results for hemagglutination, immunofluorescence and complement fixation tests. In a group of 40 patients with clinical and electrocardiographic signs of Chagas'disease, most of them showing also a positive xenodiagnosis, the hemagglutination test was positive in 100% of the cases, with titers ranging from 1/40 to 1/10,240.

INTRODUCTION

The preservation of sensitized erythrocytes is an important achievement to make hemagglutination tests practical for routine purposes. In the serologic diagnosis of American trypanosomiasis, this test is already recognized as sensitive and specific [6, 8, 10]. In this paper it is our purpose do describe the preparation and preservation of human erythrocytes sensitized with extracts of *Trypanosoma cruzi*, as performed in our laboratory. We present also comparative results between the hemagglutination test with the preserved reagent and immunofluorescence and complement fixation tests, in 1,430 serum samples collected from blood donors, known cases of Chagas' disease, and patients from a general Hospital.

MATERIAL AND METHODS

Antigens — For sensitization of cells, antigenic extracts were prepared from *T. cruzi* culture forms (Y strain)[11] obtained in a liquid medium maintained at 28°C for 5 to 7 days [4]. The parasitic growth, represented mainly by epimastigotes, was centrifuged and washed for three times in large amounts of saline solution (NaCl 0.15M), vacuum-dried at-20°C over calcium chloride or freeze-dried. One hundred milligrams of dried trypanosomes were suspended in 30 ml of saline solution and sonicated(*) for a few minutes in an ice bath. The suspension was then kept overnight at 4°C in test tubes, under continuous slow rotation. After centrifuging for 15 minutes at 2,000 x g, the sediment was discarded and about one-third volume of cold (-20°C) anhydrous ethylic ether was added to the supernatant. The mixture was vigourously shaken for 2 or 3 minutes and quickly centrifuged. The ether and interfase layers were removed. The resulting aqueous antigenic extract was kept at -20°C or freeze-dried. Protein concentrations of 1,5 to 2,0 mg/ml were usually found in such extracts, as determined by the technique of LOWRY et al. [9], with human IgG as a standard.

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Aldehyde fixation of erythrocytes — Human blood cells were fixed either with formaldehyde or glutaraldehyde. Recently collected type 0, Rh negative, bank blood samples were centrifuged and cells washed for three times with large volumes of saline solution. Formalin treatment of cells was performed according to the technique of BUTLER[3] with minor modifications. To a 10% suspension of cells in PBS (phosphate buffered saline solution: NaCl 0.15M; phosphates 0.01M; pH7.2) an equal volume of 10% formalin in PBS was slowly added, with as gentle an agitation as to avoid foaming. The misture was kept at 37°C overnight. Cells were then washed for three times in large volumes of distilled water and once in saline solution, and kept at 4°C as a 20% suspension in saline solution with 0.1% sodium azide. Usually they could be employed during periods of many months.

Occasional batches of formalin-treated cells were unsuitable for hemagglutination tests, probably when prepared from blood samples which had been kept in the refrigerator for many days before fixation, or when different formalin concentrations were used. Fixed cells should present a smooth surface and a normal morphology, with no crenation. A few unsuitable batches, when kept for weeks at 4°C and washed in saline solution, could then be used in the tests.

Glutaraldehyde fixation was based on the procedure as described by BING et al.[2]. Packed, washed cells were chilled in an ice bath and then suspended at 1% in a cold 1% solution of glutaraldehyde. This was prepared by diluting the glutaraldehyde(*) to 1% in a chilled buffer solution (4°C) prepared from the mixture of 1 volume phosphate solution (0.15M, pH 8.2), 9 volumes saline solution and 5 volumes distilled water. The glutaraldehyde cell suspension was maintained at 4°C for 30 minutes and centrifuged. Cells were washed in distilled water and in saline solution, five times each, and kept at 4°C as a 20% suspension in saline containing 0.1% azide.

Formalin or glutaraldehyde fixed cells sediment easily, forming a compact layer, which however can be homogeneously resuspended through a vigourous shaking of the flask.

Sensitization of fixed cells with antigenic extracts — Binding antigenic extracts of *T. cruzi* on fixed erythrocytes was better obtained through tannic acid or chromium chloride treatment of cells. Other techniques based on coupling through other agents, as glutaraldehyde, bis-diazotized benzidin or ethylcarbodiimide, were not as successful.

For the tannic acid treatment a mixture of equal volumes of a 2% suspension of cells and tannic acid solution (1/15,000), both in saline solution, was kept at 37°C for 40 minutes. After centrifuging and washing twice the cells were suspended at 2% in the antigenic extract. This was diluted in buffered saline solution (one volume NaCl 0.15M plus one volume 0.15M phosphate pH 7.2) so as to insure a maximal sensitization of cells, indicated by the highest titer for a positive standard serum. The suspension was kept at 37° for 50 minutes. At the end of this period formalin could be added to preserve the sensitized cells, as to be described, or the cells were washed twice and suspended at 2% in saline solution, for use.

Binding through chromium chloride was performed as described[5], by suspending erythrocytes at 2% in saline solution of the antigenic extract, diluted so as to result in maximal reactivity of the cells. For each milliliter of this suspension, 0.4 ml of a 0.8% solution of chromium chloride ($CrCl_3.6H_20$) in distilled water were added and the mixture kept at room temperature for 7 to 10 minutes. The cells were then centrifuged, washed twice and suspended at 2% in saline solution. The chromium chloride solution was prepared at the moment of use from an approximately 10% stock solution and its concentration could be easily ascertained through a colorimetric reading, since it corresponded to a transmitance of 42% in a Coleman Jr. Spectrofotometer (λ = 610 nm, 12mm cubes).

Both for tannic acid or chromium chloride techniques, maximal sensitizing antigenic dilutions usually presented protein concentrations of 30 to 50 micrograms per milliliter.

Preservation of sensitized cells — Although suspensions of sensitized fixed cells can be

(*) Glutaraldehyde solution 50% w/w, Biologic grade, Fisher Scientific Co., USA

used in the hemagglutination tests for several weeks when maintained at 4°C, preservation for much longer periods can be obtained by freeze-drying. For this purpose, to the suspension of erythrocytes in the antigenic solution, just after the incubation time necessary for sensitization, an equal volume of 6% formalin in buffered saline solution, pH 7.2, was slowly added under continuous stirring. The mixture was kept at 37°C for 3 to 4 hours under stirring, and then it was left overnight at 4°C. Cells were sedimented through centrifugation, washed in buffered saline solution and suspended at 6% in a phosphate solution (0.15M, pH7.2) containing 6% normal rabbit serum. After distributing in ampoules in volumes of 1 ml to 3 ml, the suspension was shell-frozen at -70°C in a mixture of alcohol-dry ice and freeze-dried for 8 to 16 hours (0.005mm Hg, sublimation at room temperature, condensation at -70°C).

For use, suspensions were reconstituded to the original volume with a solution of 0.5% Tween 80(*) in distilled water containing 0.3% sodium azide, and diluted by the addition of two volumes of saline solution.

Hemagglutination test — In microtiter plastic plates(**) with V-shaped wells, one drop (0.025 ml) of the antigenic suspension of sensitized cells was added to two drops (0.05 ml) of a 1:40 dilution of serum in buffered saline solution (NaCl 0.15M; phosphates 0.05M, pH 7.2, 2% rabbit normal serum). For quantitative tests, doubling dilutions from 1:40 on were used. Plates were carefully shaken to mix reagents and left at room temperature. Tests could be read after 1 or 2 hours or even next day, following habitual criteria for positive and negative results [5].

It was possible to dilute serum samples directly in microtiter plates. Two drops of diluent were added to wells in three successive rows. By tranferring serum with a 0.025 ml loop to the first and then to a second and a third wells a 1:27 dilution was obtained in the last one, sufficient for a qualitative test.

Immunofluorescence and complement fixation tests — Immunofluorescence tests were performed as described [4], with *T. cruzi* epimastigotes as antigen and an anti-human-IgG fluorescein conjugate showing a F/P weight ratio of 7, diluted so as to contain at least 1/4 antibody precipitating unit [1]. Complement fixation tests followed the technique of FREITAS [7].

Serum samples — A total of 1,430 serum samples were studied including 3 from patients with acute forms of Chagas'disease, 40 from chronic forms showing clinical and electrocardiographic signs of the disease, most of them presenting also a positive xenodiagnosis test. The remaining sera were collected from blood bank donors, or patients sent for routine serologic diagnosis. Sera were tested the same day of collecting or otherwise kept at -20°C until use. Before testing, samples were decomplemented at 56°C for 30 minutes.

RESULTS

The hemagglutination test was positive for all the 40 serum samples from chronic cases of Chagas'disease. Titers ranged from 1:40 to 1:10,240, the majority of which showing values from 1:320 to 1:2,560. Immunofluorescence and complement fixation tests were also positive for these cases. The 3 acute cases of Chagas'disease were fluorescence positive, complement fixation negative, but in only one a positive hemagglutination tests was found. Table I shows comparative results between hemagglutination and immunofluorescence tests for the sera here studied, from which only the 3 samples from acute cases of Chagas'disease were excluded. A strait correlation was found between both tests; which presented similar results in 1,421 cases (99.6%). Only 6 cases had divergent results, and for these the complement fixation test resulted negative.

When comparing hemagglutination and complement fixation tests (Table II), a tight correlation was also observed. A concordance was found for 1,399 serum samples, corresponding to 99.1% of those for which a conclusive complement fixation result could be obtained. Most of sera presenting divergent results in both tests had positive hemagglutination, negative complement fixation tests.

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TABLE I

Comparative results between hemagglutination and immunofluorescence tests for American trypanosomiasis, in 1,427 serum samples.

Hemagglutination test	Immunofluorescence test			Total
	Reactive	Non-reactive	Doubtful	
Reactive	158	3	0	161
Non-reactive	2	1,262	0	1,264
Doubtful	1	0	1	2
Total	161	1,265	1	1,427

TABLE II

Comparative results between hemagglutination and complement fixation tests for American trypanosomiasis, in 1,427 serum samples.

Hemagglutination test	Complement fixation test			Total
	Reactive	Non-reactive	Doubtful or anti-complementary	
Reactive	143	9	9	161
Non-reactive	1	1,256	7	1,264
Doubtful	0	2	0	2
Total	144	1,267	16	1,427

Freeze-dried cells could be maintained at 4°C for at least one year (the largest period of observation). Even when reconstituted, resulting suspensions could be kept for 2 or 3 months in the refrigerator, with no evidences of a diminished antigenic activity.

DISCUSSION AND CONCLUSIONS

Results here presented indicate the hemagglutination test for American trypanosomiasis with preserved cells as sensitive and specific a test as the immunofluorescence and complement fixation procedures.

Stability of sensitized cells was satisfactory and thus it permits reagents to be prepared in central laboratories and distributed to those directly interested in routine work. In this way, not only the hemagglutination test can be easily performed in clinical laboratories or blood banks, but also a better standardization of reagents and results can be attained than when fresh cells are used.

The hemagglutination test can thus be recommended not only as a screening test but also to be performed in parallel with any other serologic test for the diagnosis of *T. cruzi* infection. As in other diagnostic procedures, the concomitant running of more

than one test increases the reliability of the results, since tests can thus be checked by each other. The short time necessary for obtaining results, facility to perform and clear cut patterns for positive and negative results, make the hemagglutination test a valuable procedure for the serologic diagnosis of *Trypanosoma cruzi* infections.

RESUMO

Reação de hemaglutinação com glóbulos sensibilizados e preservados: técnica prática para o diagnóstico sorológico da tripanosomíase americana

Descrevem-se com minúcias as técnicas utilizadas para a sensibilização por extratos de *T. cruzi*, e posterior preservação, de hemácias humanas fixadas por aldeídos. A estabilidade de tal reagente torna a reação de hemaglutinação muito prática para fins de rotina, no diagnóstico sorológico da tripanosomíase americana. Além de facilitar sobremaneira a execução da prova, o emprego de eritrócitos preservados facilita a padronização da técnica.

O estudo comparativo entre as reações de hemaglutinação, imunofluorescência e fixação do complemento, em 1430 soros, mostrou estreita correlação entre essas três provas, realizadas segundo as técnicas propugnadas.

De 40 pacientes com diagnóstico de doença de Chagas, na sua maioria com evidenciação do parasita, todos apresentaram reação de hemaglutinação positiva, em títulos que variaram de 1/40 a 1/10.240.

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