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A STABLE POLYSACCHARIDE-HEMAGGLUTINATION REAGENT FOR THE DIAGNOSIS OF ACUTE OR RECENT TRYPANOSOMA CRUZI INFECTIONS (*)

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SUMMARY

A stable hemagglutination reagent was developed for antibodies against **Trypanosoma cruzi** polysaccharide components. Conditions for sensitizing aldehyde-fixed human erythrocytes with parasite polysaccharides are described, as well as for obtaining a lyophilized reagent. Hemagglutination of polysaccharide-sensitized cells was shown to be due to IgM antibodies. These were observed in every case studied of acute Chagas'disease, however they were absent or in very low titers in chronic cases. Possible seroepidemiologic applications of the test are considered.

INTRODUCTION

Sera from patients with acute or recent Chagas'disease were shown to react with polysaccharide components of **Trypanosoma cruzi** both in precipitin and complement fixation tests [8,9]. Preliminary evaluation of such antigens in the precipitin tests presented 97.9% to 100% positives in acute cases and 17% to 18% in chronic infections [9,12]. Also in the complement fixation test, polysaccharide antigens furnished higher titers for acute than for chronic cases, only a few of which showed positive results [9,11].

The hemagglutination test with stable, preserved reagents, is a very practical procedure for Chagas'disease routine serological diagnosis, both for clinical and seroepidemiological purposes. However, the usual test with a reagent prepared by coating with **T. cruzi** extracts aldehyde-fixed cells treated by tannic acid or chromium chloride, although showing a high sensitivity for chronic cases [8], is of limited diagnostic value for recent infections, where low titers or negative results are common.

Recently ANTUNES et al. [1] have described an hemagglutination test for muco-cutaneous leishmaniasis, with a **Leishmania brasiliensis** polysaccharide antigen fixed on fresh red cells. Positive results were observed for 91% of the tested cases and only low-titered cross-reactions were seen with sera from Chagas'disease patients. However, when trying to sensitize erythrocytes with **T. cruzi** polysaccharide components, the techniques described for coating both fresh [1] and aldehyde-treated [5] cells with polysaccharide antigens were not successful. This paper presents the standardization of a polysaccharide-hemagglutination test for Chagas'disease and results obtained with a few sera from acute or chronic Chagas'infection.

MATERIAL AND METHODS

T. cruzi antigens

a) **Soluble saline extract** — A suspension of 100 mg lyophilized **T. cruzi** epimastigotes in 30 ml saline solution was desintegrated by ultrasonic oscillation for 2 minutes, in an ice-bath, and kept overnight at 4°C in a rotator. After centrifuging at 10,000 x g for 30 minutes at 4°C, the supernatant was stored at -70°C until used.

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b) **Saline-insoluble fraction** — The pellet was resuspended in a large volume of saline solution and kept in a rotator at 4°C for 24 hours, with 3 changes of saline solution in order to remove all soluble components. To the washed pellet, 15 ml of 0.15 M NaOH solution were added and the mixture kept at 4°C for 18 hours. After neutralizing with an equal volume of 0.15 M HCl solution, eventual insoluble particles were removed by centrifugation of the mixture and the supernatant was kept at -70°C until used.

c) **Whole crude antigen** — One hundred milligrams lyophilized **T. cruzi** epimastigotes were mixed with 15 ml 0.15M NaOH, in a tissue homogenizer, and kept overnight at 4°C. After neutralizing with HCl solution, any insoluble residue was removed by centrifugation.

d) **Polysaccharide antigens** — Parasite polysaccharide fractions were prepared according to the techniques described by MUNIZ & FREITAS [9], GONZALEZ & YAMAHA [4] and NASH et al. [10].

Protein and polysaccharide determinations

Proteins were determined according to LOWRY et al. [6] and polysaccharides according to MARTIRANI et al. [7], in the different antigens.

Formalin-fixed erythrocytes

Freshly collected human group 0, Rh-negative erythrocytes were washed 4 times with a 0.15M NaCl solution and suspended at 10% in PBS (0.15 M NaCl, 0.01 M phosphates, pH 7.2). An equal volume of 4% formaldehyde solution in PBS was added and the mixture kept at 37°C for 18 hours, with occasional stirring. Cells were washed 3 times in distilled water or saline solution and suspended at 20% in 0.15 NaCl solution with 0.8% formaldehyde. Such formalin-fixed cells could be kept at 4°C for at least 2 years with no apparent deterioration.

Sensitization of formalin-fixed erythrocytes with T. cruzi antigens

a) **Polysaccharide antigens** — Cells were incubated for varying periods with polysaccharide antigens diluted in different solutions at different temperatures and pH values. To the suspension was then added an equal volume of 0.1% glutaraldehyde saline solution, the mixture incubated for 30 minutes at 37°C. After washing twice with saline solution, the cells were suspended for immediate use at 2% in PBS pH 6.4 containing 0.9% dried skim milk.

b) **Protein antigens** — The procedure described by CAMARGO et al. [2] was followed, with minor modifications. In brief, same volumes of a 2% cells suspension and a 1:15,000 tannic acid dilution, both in saline solution, were mixed and incubated at 56°C for 15 minutes. Cells were washed twice in saline solution and resuspended at 2% in antigen diluted in PBS pH 6.4. After incubating at 37°C for 50 minutes, an equal volume of 0.1% glutaraldehyde in PBS pH 6.4 was added to the suspension and the mixture maintained at 37°C for 30 more minutes. After washing for three times in saline solution, cells were suspended at 2% in PBS 6.4 containing 0.9% dried skim milk, for immediate use.

Preservation and storage of hemagglutination reagents

Sensitized erythrocytes were suspended at 6% in a preserving solution (PBS pH 6.4 containing 4% glycine, 2.7% dried skim milk, 2% sodium glutamate, 0.1% sodium thioglycolate and 0.1% merthiolate) and lyophilized in 1.0 ml aliquots. For use each aliquot was reconstituted with 3.0 ml distilled water. Reconstituted reagent could be kept at 4°C for at least two months with no loss of activity.

Serologic tests

Passive hemagglutination, complement fixation, anti-IgG immunofluorescence and anti-IgM immunofluorescence tests were performed as already described [2].

Serum samples

As soon as parasitemia was detected in patients with acute Chagas'disease, serum samples were collected at different successive intervals. These were mostly post-transfusional patients.

Sera were also obtained from chronic cases of Chagas'disease and from non-infected individuals.

Treatment of sera with 2-mercaptoethanol

The technique of DEUTSCH & NORTON [3] was followed for treating sera with 2-ME.

RESULTS

Standardization of fixed-erythrocytes polysaccharide sensitization

When incubating aldehyde-fixed cells under variable conditions of temperature and time with **T. cruzi** polysaccharide antigens diluted in a series of different solutions and buffers with a wide pH range, it was observed that only alkaline NaOH antigenic solutions were able to sensitize the cells.

Optimal conditions were investigated first with extracts prepared according to NASH et al. [10], but similar results were later found for parasite fractions obtained as described by MUNIZ & FREITAS [9] and by GONÇALVES & YAMAHA [4]. Strikingly, it became patent that previous isolation of such components was not necessary, since at optimal conditions sensitization results were the same with either purified polysaccharides or **T. cruzi** total extracts. In Table I results of sensitizations both with purified components or total extract, for different NaOH concentrations, are expressed by test titers of a serum sample from an acute case of Chagas'disease. Results were negative for sera from chronic cases and from non-infected individuals.

TABLE I

Serum titers for antigens diluted in different NaOH solutions (sensitization at 56°C for 15 minutes)

Antigens	NaOH concentration:							
	2M	1M	0.5M	0.2M	0.1M	0.05M	0.02M	0.01M
Polysaccharide — (10 μg/ml)	640	1,280	2,560	1,280	1,280	640	—	—
Total extract (Polysaccharide — 10 μg/ml Protein — 170 μg/ml)	640	2,560	1,280	2,560	1,280	1,280	640	—

It was observed that cell coating with polysaccharide antigens could be obtained in a temperature range of 4°C to 56°C, as long as suitable incubation was allowed. Longer periods were required at lower temperatures but, at 56°C, 5 minutes were enough for maximal sensitization. No increase in titers were observed when incubation was lengthened to as much as 60 minutes.

The study of antigen concentration showed that maximum sensitization could be obtained with as low as 3 μg/ml polysaccharide (Table II).

TABLE II

Serum titers for different polysaccharide antigen concentrations (sensitization in NaOH 0.5 M, for 10 minutes at 56°C)

Antigens	Polysaccharide concentration (sugar contents μg/ml)						
	25.0	12.5	6.0	3.0	1.6	0.8	0.4
Purified fraction	1,280	2,560	1,280	1,280	640	640	160
Total extract	2,560	2,560	1,280	1,280	1,280	640	320

Yield and antigenic activity of polysaccharide components from T. cruzi extracts or fractions

For 100 mg lyophilized trypanosomes, a total 2.14 mg polysaccharides could be obtained from the crude **T. cruzi** extract by the technique of NASH et al. [10]. Such amounts were respectively 0.68 mg and 1.22 mg for the saline-soluble and for the saline-insoluble fractions.

Sensitization patterns and antigenic activities were similar for polysaccharide components obtained from either **T. cruzi** crude extract or its fractions.

Heat-stability of T. cruzi polysaccharide antigens

No differences were observed for sensitizing patterns or antigenic activity of **T. cruzi** polysaccharide components after boiling for 1 hour in a water-bath or autoclaving at 120°C for 30 minutes.

Treatment of sera with 2-mercaptoethanol

Serum samples from acute and chronic cases of Chagas'disease were submitted to different serologic tests, before and after treatment by 2-ME. The tests included anti-IgG and anti-IgM immunofluorescence, complement fixation, protein-hemagglutination and polysaccharide-hemagglutination tests. Results shown in Table III indicate polysaccharide-hemagglutination to depend on antibodies which are reduced by 2-ME.

TABLE III

Serum titers for cases of recent and chronic Chagas' infection before and after treating samples with 2-mercaptoethanol

	Test titers							
	Before 2-ME treatment				After 2-ME treatment			
	Poly HA	Prot HA	IgM-IF	IgG-IF (*)	Poly HA	Prot HA	IgM-IF	IgG-IF
Acute cases								
A1	640	160	160	160	0	0	0	160
A2	320	80	160	160	0	0	0	160
A3	320	160	320	160	0	0	0	80
A4	640	0	160	160	0	0	0	320
A5	320	160	160	320	0	0	0	320
A6	160	80	160	320	0	0	0	160
Chronic cases								
C1	40	1,280	0	640	0	1,280	0	640
C2	0	1,280	0	640	0	1,280	0	320
C3	0	640	0	320	0	1,280	0	320
C4	0	640	0	160	0	640	0	160
C5	20	2,560	0	160	0	2,560	0	160
C6	0	2,560	0	320	0	1,280	0	160

(*) PolyHA — polysaccharide hemagglutination test
ProtHA — protein hemagglutination test
IgM-IF — immunofluorescence test for IgM antibodies
IgG-IF — immunofluorescence test for IgG antibodies

Results of the polysaccharide hemagglutination tests in T. cruzi infected and non-infected patients

Serum samples from 44 non-infected individuals taken at random were tested, and 40 showed negative results while in the remaining 4 a 1:40 titer was observed.

Thirty nine chronic Chagas'infection cases were studied. Positive tests were seen in 5 cases, 4 at 1:40 and 1 at 1:80, with 34 nega-

tives, whereas much higher titers were detected in all 12 cases in the acute phase of the disease. Thus, titers of 1:80 and 1:160 were observed only in two cases. The others showed titers of 1:320 (2 cases), 1:640 (1 case), 1:1,280 (4 cases) and 1:2,560 (3 cases).

Periods after infection during which the polysaccharide test remains positive could not be determined, although this could be an important information for seroepidemiological applications. Most of our acute cases have been submitted to chemotherapy and in two, negativation was observed for all serologic tests performed, including the polysaccharide hemagglutination test. For both cases, this test became negative respectively 15 and 36 months after the infection initiated. For other 7 cases of acute infection, polysaccharide hemagglutination test, as well as the other serologic tests, are still positive after periods varying from 3 to 31 months.

DISCUSSION

In endemic areas of American trypanosomiasis, recognizing recent **T. cruzi** infections presents a difficult problem, both on clinical and laboratory grounds. Antibodies against polysaccharide components of the parasite are known to occur in acute Chagas'disease but only occasionally in chronic cases. However available tests are not satisfactory for seroepidemiology purposes. Development of stable, lyophilized, human cells sensitized with **T. cruzi** polysaccharide components introduces new possibilities. Besides very practical for routine purposes, the hemagglutination test can be performed both for serum samples and for eluates from blood samples dried on filter paper. Our preliminary results confirm the described occurrence of antibodies against **T. cruzi** polysaccharide antigens in recent Chagas'infections. However, investigations are necessary to evaluate the new hemagglutination test for seroepidemiological applications.

RESUMO

Reagente estável para teste de hemaglutinação com antígenos polissacarídicos do T. cruzi, para diagnóstico de infecção chagásica aguda

Descreve-se reação de hemaglutinação para anticorpos antipolissacárides do **T. cruzi**, definindo-se condições para preparo de reagente estável, liofilizado. Demonstrou-se que tais anticorpos pertencem à classe IgM. O teste foi positivo em todos os casos de infecção chagásica aguda estudados, mas negativo ou com títulos muito baixos nos casos crônicos. Estes resultados sugerem a possibilidade de aplicação do teste para estudos soroepidemiológicos de incidência da infecção.

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