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PLASMA MEMBRANE ORIGIN OF TRYPANOSOMA CRUZI ANTIGENIC DETERMINANTS IN CHAGAS' DISEASE

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SUMMARY

Passive hemagglutination tests, using human erythrocytes coated with either **T. cruzi** antigens extracted from whole cell epimastigotes or antigens from purified epimastigote plasma membrane, have been performed with sera from 140 human patients of Chagas' disease. All cases were positive with both antigens, while sera from 188 non-infected humans were unreactive. Both antigens behave similarly, even with regards to their cross-reactivity with sera from other infectious diseases. In order to obtain maximal response with standard serum of Chagasic patients, the minimal amount of input protein necessary to saturate the erythrocytes surface (1.2 μg for 2.34 x 10^8 cells) was found to be 7-10 fold less for the plasma membrane antigen as compared to the whole cell antigen. Since plasma membranes were likewise shown to have been purified 10 fold, as measured by the net enrichment of two independent membrane markers, it is concluded that the majority of the antibodies directed against trypanosomes in natural human infections are elicited by membrane-bound antigens, most of which are common to both the trypo and epimastigote forms.

INTRODUCTION

The passive hemagglutination test, which is widely employed for serological diagnosis of human Chagas'disease, has many advantages, including its relative simplicity and its reasonable high sensitivity.

Recently, we [22] have obtained a fraction of isolated plasma membrane from epimastigote forms to **T. cruzi.** In the present work, we report a comparative study of the passive of hemagglutination elicited by sera from patients affected with Chagas' disease using erythrocytes coated with either total antigen from epimastigotes or antigens from our purified plasma membrane fraction. The results suggest that part of the circulating antibodies in such patients are raised against membrane determinants, most of which are common to the trypo and epimastigote forms.

MATERIALS AND METHODS

Cells — Epimastigote forms, Y strain [17], were cultured at 28°C in LIT medium [3] at 120 rev/min in a rotatory shaker. Cells were collected by centrifugation (1,000 x g) during late exponential growth (80-100 h) and washed 3 times with 0.9% (w/v) NaCl solution. The isolation of plasma membrane fractions from epimastigotes was accomplished essentially as described [22]. Briefly, cells were disrupted by sonication under carefully controlled conditions and the homogenate was subjected to differential centrifugation. The fraction sedimenting at 14,000 x g was further purified by sucrose gradient centrifugation. The gradient fractions enriched in adenylyl cyclase were pooled and sedimented by centrifugation at 80,000 x g for 1 h at 4°C. This fraction is 10-15 fold enriched in both adenylyl cyclase and in bound iodine.

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Bound iodine was introduced by radioiodination of the intact epimastigote forms with $Na^{125}I$ by lactoperoxidase, as described [22]. Radioactivity was determined in a gamma-counter with an efficiency of 20%. No significant contamination with other cellular organelles could be detected in these fractions.

Antigen preparation — Antigens were prepared essentially according to CAMARGO et al. [2], with only minor modifications. Washed epimastigote forms (20 mg wet weight) were resuspended in 5 ml of 0.15 N NaOH and sonicated in a Branson sonifier cell disruptor. The homogeneous suspension was maintained for 6 h at 4°C and then neutralized with HCl. Thioglycolate (6 mg) and merthiolate (6 mg) were added and the solution allowed to stand overnight at 4.°C. Antigen from whole cells, hereafter denominated total antigen (TA), was recovered in the 10,000 x g supernatant. The antigen from the purified plasma membrane fraction (MA) was obtained by the same procedure, except that sonication was performed using 1 mg of plasma membrane protein in 5 ml of 0.15 N NaOH.

Hemagglutination test — Sensitization of human O Rh— red blood cells was performed according to CAMARGO et al. [2] using TA and MA. Lyophilized sensitized red blood cells retained their ability to agglutinate even after 3 months of storage at 4°C. The test was standardized with control serum from Chagasic patients. Positivity was significant with serum dilutions above 1/4 in the case of erythrocytes coated with MA and above 1/20 when TA was employed. Sera were collected from ambulatory patients from the City of São Paulo and stored in small aliquots at -20°C.

Additional tests — Sera were obtained from patients with clinical diagnosis of Chagas' disease, toxoplasmosis, cutaneous leishmaniasis, syphilis, schistosomiasis and cysticercosis, and further characterized through specific serological tests. The Machado-Guerreiro complement fixation reaction [13], using soluble antigen prepared according to MAEKELT [11], and indirect immunofluorescence tests [1] were used in the diagnosis of Chagas' disease. Toxoplasmosis was characterized by immunofluorescence tests, using as fluorescent conjugates antisera against human IgG and IgM [6]. Cutaneous leishmaniasis was detected by indirect immunofluorescence [5]. Schistosomiasis was confirmed by immunofluorescence tests in thin slices from livers of infected mice [4,21]. The tests employed to confirm syphilis were complement fixation as described [8,9], VDRL agglutination [20], and FTABs [5]. Cysticercosis was confirmed by complement fixation [18], using method II for antigen extraction.

Protein contents in the antigens — Proteins was determined by the method of LOWRY et al .[10], using bovine serum albumin as standard.

RESULTS

Hemagglutination tests with sera from Chagasic patients

Passive hemagglutination tests were performed with sera from 140 patients with chronic Chagas' disease. In addition, parallel complement fixation and indirect immunofluorescence tests were carried out on all sera. Furthermore, all sera were positive with erythrocytes coated with both TA and MA. With TA, 9 cases were positive with serum dilutions of 1/40-1/80, 47 were positive with titers between 1/160-1/320 and 84 were positive with higher titers. With MA, 10 cases were positive with titers between 1/8-1/16, 72 were positive with titers between 1/32-1/64, and 58 were positive with higher titers (Table I). Serum samples from 188 non-Chagasic patients were negative with both TA and MA-coated erythrocytes. Indirect immunofluorescence and complement fixation tests were used in all cases to confirm normality or infection.

Cross-Reactivity

Passive hemagglutination with TA and MA-coated erythrocytes was checked using sera from patients with several other parasitic diseases (Table II). Both TA and MA antigens cross-

TABLE I

Titers of human sera from chagasic patients in the passive hemagglutination test using TA (whole cell epimastigotes) and MA (purified plasma membranes from epimastigotes) coated erythrocytes

TA	Number of sera tested	MA	Number of sera tested
1/40 — 1/80	9	1/8 — 1/16	10
1/160 — 1/320	47	1/32 — 1/64	72
> 1/320	84	> 1/64	58

TABLE II

Passive hemagglutination elicited by sera from humans with different parasitic diseases using TA (whole cell epimastigotes) and MA (purified plasma membranes from epimastigotes) coated erythrocytes

	Number of	Positivity (%)		Co-negativity
	cases	TA	MA	RATIO
Normal	188	0	0	1.0
Chagas	140	100	100	1.0
Toxoplasmosis	57	1.7	7	0.946
Syphilis	61	0	1.6	0.948
Cutaneous Leishmaniasis	17	11.8	11.8	1.0
Schistosomiasis	10	20	60	0.5
Cysticercosis	17	5.9	41.1	0.625

reacted to a variable extent with antibodies raised against these other pathogenic agents. Significant cross-reactivity has been observed only with schistosomiasis and cysticercosis. This can be seen by the co-negativity ratios which reflect the degree of concordance between the results obtained with TA and MA antigens (Table II). Thus toxoplasmosis, syphilis and cutaneous leishmaniasis gave co-negativity ratios approaching 1. In contrast, the ratios for schistosomiasis and cysticercosis are significantly different from this unity particularly with MA, possible because this fraction is enriched in antigens common to the three parasites. It should be noted that these cross-reactivities obtained in the hemagglutination tests were absent in the indirect immunofluorescence tests with **T. cruzi** antigens, with the exception for leishmaniasis.

Measurement of antigen-protein bound to sensitized erythrocytes

The amount of protein bound to a given number of erythrocytes using either TA ou MA was estimated as follows: the cell surface of epimastigote forms of **T. cruzi** was labelled with $Na^{125}I$ by the lactoperoxidase catalyzed reaction [22]. Half of the labelled cells were used to prepare purified plasma membranes. TA and MA were prepared from radioactive cells and purified plasma membranes, respectively, as described in **Materials and Methods.** Increasing amounts of antigen protein were used to coat 2.34 x 10^8 erythrocytes. In each case, the amount of protein chosen to establish comparison was the minimal amount of protein necessary to give maximal reactivity in hemagglutination tests with standard sera from Chagasic patients. In the case of TA, it was necessary to use 84.5 μg of protein (S.A. = 8.3 x 10^5 cpm/mg protein) in order to coat the erythrocytes with 1.18 μg of protein, as measured by the amount of bound radioactivity. This represents 1.4% of the total protein used to coat the erythrocytes at saturation. In the case of MA, 11.9 μg of protein (S.A. = 22.5 x 10^5 cpm/mg protein) was required to bind 1.15 μg to the same number of erythrocytes, a value which corresponds to 9.7% of the total protein input. These data show that 7-fold less protein is necessary for maximal coating of 2.34 x 10^8 erythrocytes when MA is used as compared to TA. With several other preparations of plasma membrane, this ratio approached 1/10, which is the estimated purification obtained for the plasma membrane preparation, as judged by two independent markers, i.e., adenylyl cyclase activity and bound labeled iodine [22].

Specific location of the antigens

Identical tests have been performed with cytosolic proteins from epimastigote forms obtained by centrifugation of the homogenate at 105,000 x g after disruption by sonication. Using this cytosol, no hemagglutination was observed with sera from several patients with chronic Chagas' disease.

DISCUSSION

Both we [22] and others [12,14,15,16,19] have been involved in the purification of subcellular fractions from **T. cruzi.** Although several subcellular fractions can elicit immunological response in experimental animals [14,19], only the plasma membrane fraction and the flagellar fraction show some protective activity against experimental **T. cruzi** infections [15,16].

This latter observation is confirmed by the present results, which demonstrate that antigens extracted from the plasma membrane are capable of reacting with circulating antibodies from chronic Chagasic patients. Passive hemagglutination promoted by sera of 140 affected persons gave a strict correlation between antigens extracted from whole epimastigotes or from purified epimastigote plasma membranes. When the test was applied to sera from pa-

tients with other parasitic diseases both antigens paralleled in cross-reactivity, except in the case of cysticercosis and schistosomiasis, where plasma membrane antigens were more reactive, possibly due to the concentration of common antigens in this fraction. The amount of antigen protein necessary to coat the erythrocytes surface and elicit the same reactivity to a standard Chagasic serum was 10-fold less when antigens of plasma membrane origin were employed, as compared to antigens from whole cell epimastigotes. This factor correlates well with the estimated enrichment of the plasma membrane fraction, as measured by the adenylyl cyclase and bound iodine specific activities [22]. Clearly, then, the antigenic determinants which elicit humoral immunological response in chronic Chagasic patients co-purify with independent membrane markers.

When erythrocytes were coated with the cytosolic fraction, using the same experimental procedure, no hemagglutination was observed with sera from chronic Chagasic patients. This result is at variance with the report [14] of antibodies against cell sap proteins in infected animals and Chagasic humans, using complement fixation tests. A possible explanation for this discrepancy is that those authors did not compare their results by the passive hemagglutination test and used cell sap from trypo and amastigotes. On the other hand, SEGURA et al. [15] have unequivocally shown that no protective activity against **T. cruzi** infection is observed when mice are immunized with cell sap proteins from epimastigotes. Indeed, only when plasma membrane and flagellar fractions were used as immunizing agents was a high protection level obtained [15,16].

It is important to emphasize that in the present work we were able to quantify the amount of antigen bound to the erythrocyte surface by labeling of the cell surface prior to antigen extraction. Although the procedure employed in this work is limited somewhat by the loss of polysaccharide during antigen preparation [7], this methodological approach should be adopted whenever reactions involving coating of erythrocytes with proteic antigens are performed.

Finally, considering that it is the trypomastigote, and not the epimastigote that is responsible for a natural infection, our results suggest that a considerable proportion of the antigenic determinants common to both cell forms are membrane located.

RESUMO

Determinantes antigênicas de membrana plasmática do Trypanosoma cruzi **na doença de Chagas**

Examinaram-se soros de 140 pacientes portadores de Doença de Chagas por meio de testes de hemaglutinação passiva usando-se eritrócitos humanos encapados com antígenos extraídos de formas epimastigotas de **T. cruzi** ou de membranas plasmáticas purificadas dessas formas. Todos os casos foram positivos com ambos os antígenos, enquanto os soros de 188 humanos não infectados foram negativos. Ambos os antígenos comportaram-se de forma semelhante inclusive quanto à reatividade cruzada com soros de outras doenças infecciosas. A quantidade mínima de proteína necessária para saturar a superfície dos eritrócitos (1,2 μg para 2 34 x 10^8 células) foi 7-10 vezes menor para o antígeno de membrana plasmática quando comparado ao antígeno da célula total, para reatividade máxima com soro padrão de pacientes chagásicos. Considerando-se que essas membranas estejam purificadas por um fator de 10, como se pode verificar pela medida de marcadores bioquímicos independentes, concluiu-se que a maioria dos anticorpos dirigidos contra tripanossomos em infecções humanas são induzidos por antígenos ligados à membrana plasmática, antígenos esses comuns às formas epi e tripomastigotas.

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