Rev. Inst. Med. trop. São Paulo
18(6):433-439, novembro/dezembro, 1976

UDC 593.161.13

IMMUNOLOGY OF CHAGAS' DISEASE I — CIRCULATING ANTIGENS IN MICE EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA CRUZI*

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SUMMARY

Mice acutely infected with *Trypanosoma cruzi* have in their plasma circulating antigens (exoantigens) produced by the parasite. These antigens are capable of reacting against serum of mice chronically infected with *T. cruzi* in the complement fixation and counterimmunoelectrophoresis tests but not in the haemagglutination test. Absorption of the chronic serum with culture forms of *T. cruzi* completely removed the antibody which reacts against the circulating antigens. Serum from humans chronically infected with *T. cruzi* also reacted against the circulating antigens which suggests that these antigens are also produced in human infections.

INTRODUCTION

Soluble antigens of parasite origin in the blood of the host have been described in several infections by protozoa and helminths [1, 3, 7, 8, 12, 13, 14, 20, 26]. The first report of soluble antigens in infections with trypanosomes was made by WEITZ [26] in the serum of rats infected with *Trypanosoma brucei*. This Author called such substances "exoantigens" in view of their immunogenic properties and absence of toxicity. In South American Trypanosomiasis caused by *Trypanosoma cruzi*, soluble antigens have been demonstrated in supernatant of liquid medium in which trypanosomes were cultivated [25], and in the serum of infected rats [24]. This last finding however has been awaiting confirmation.

The present report describes the presence of soluble antigens in the plasma of mice infected with *T. cruzi*, thus confirming and extending the observations of the latter Authors [24].

MATERIAL AND METHODS

Trypanosoma cruzi — Mice infected with strains Y and F1 of *T. cruzi* were employed. Strain Y was described by SILVA & NUSSENZWEIG [23] and F1, isolated from a naturally infected *Triatoma infestans*, was studied by BRENER [4]. Both strains are virulent for mice and present an interesting peculiarity regarding blood stream forms. In strain Y the blood stream forms at the peak of parasitemia are almost totaly represented by slender trypanosomes whereas in strain F1 they are mostly stout. The significance of this morphological variation has not been elucidated yet [5]. Plasma of acutely infected and serum of chronic animals parasitized with strain Y were used. From mice infected with the F1 strain only plasma of acute animals was employed.

Plasma from acutely infected mice — Mice infected with both strains were bled 7 days after infection when parasitemia is approximately 5 x 10^4 trypanosomes per mm^3. Both strains are maintained in our labora-

Trabalho realizado dentro do convênio existente entre a UFMG e o Centro de Pesquisas "Renê Rachou", FIOCRUZ, INERu e com auxílio do Conselho Nacional de Pesquisas.

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tory through passage into mice performed weekly. Blood was collected with heparin or citrate, and immediately centrifuged at 1,000 rpm for 5 minutes in a refrigerated centrifuge. The supernatant was carefully removed and centrifuged at 15,000 rpm for 30 minutes at 4°C to sediment trypanosomes. After this procedure the supernate was filtered through a millipore filter (pore size 0.2 μ). Plasma was concentrated 5 times using Aquacide (Calbiochem, San Diego, Calif.), dialyzed against PBS, centrifuged, and kept frozen at-20C. An aliquot of this material was used to precipitate the gamma globulins using a 33% saturated ammonium sulphate solution. The gamma globulins and the supernate from the precipitation procedure were dialyzed with distilled water. Removal of the gamma globulins was determined through immunoelectrophoresis.

Serum from chronically infected mice — Chronically infected mice were bled without anticoagulant. Serum was separated by centrifugation, filtered through millipore filter and stored at-20C. Infection in these mice was of at least 3 months duration. Some had been reinfected with organisms from the same strain. Parasitemia was very low or absent in these animals.

Complement fixation test (CF) — This test was performed according to previously described methods [9]. Concentrated and non-concentrated whole plasma, precipitated gamma globulins, and supernate from the ammonium sulphate precipitated plasma were employed as a source of antigen. All were inactivated for 60 minutes at 56°C just prior to the reaction and tested undiluted, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160. Plasma of normal mice was used as control. Sera from humans known to be parasitized with *T. cruzi* were used as a source of antibody. Recommended controls for this test were used routinely.

Hemagglutination test (HA) — This test was performed using a modification of the technique described by CAMPBELL et al. [6]. Antigen to sensitize human erythrocytes of the 0 Rh negative group was concentrated plasma and supernate from the ammonium sulphate precipitation.

Counterimmunoelectrophoresis (CE) — A modification of the technique described for amoebiasis was employed [18], 2.5 x 7.5 cm microscope slides were covered with 3 ml of 0.8% agarose (Merck Co.) in 0.05 M veronal buffer, pH 8.6. The same buffer was used in the electrophoresis chamber. Wells were usually 4 mm diameter and 6 mm apart. Serum from chronically infected mice was applied into the anode well and plasma of acutely infected mice into the cathode well. Electrophoresis was performed for a period of 90 minutes at room temperature employing 5 Volts/cm² with a Shandon SAE 2161 power unit. After the test the slides were washed 4 days with saline and distilled water, dried, and stained with Ponceau's stain.

Absorption of serum — To remove antibody against circulating antigen from serum of chronically infected mice an aliquot of this serum was absorbed with packed live culture forms of *T. cruzi* for 60 minutes at room temperature followed by an overnight stay at 4°C. After absorption the serum was centrifuged at high speed and the supernatant used in the counterimmunoelectrophoresis test.

RESULTS

Complement fixation — Gamma globulin as antigen did not fix complement at any dilution whereas the supernatant did up to a dilution of 1:80. Both Y and F1 concentrated plasma fixed complement even when diluted 1:80. When non concentrated plasma was employed the test yielded positive results at a lower dilution. Plasma from uninfected mice usually did not fix complement; however in a few tests some samples gave positive fixation when tested undiluted.

Haemagglutination — The same substances used as antigens in the CF test were used to sensitize erythrocytes for the HA test. However the HA tests were consistently negative. Antigen prepared from culture forms of *T. cruzi* was used as control with good results.

Counterimmunoelectrophoresis — In this test concentrated and non-concentrated plas-

ma of mice acutely infected with both strains of *T. cruzi* were used as source of circulating antigen. Serum from mice chronically infected with the Y strain was employed as source of antibody. The results of the CE test are shown in Figures 1, 2, 3, 4, and 5. At least one well stained and one diffuse precipitin lines were clearly visible between the wells where plasma Y and serum Y were applied (Fig. 1). When plasma F1 reacted with serum Y at least two precipitin lines formed (Fig. 2). This was noted with all 4 batches of plasma F1 employed in this study. Plasma from normal mice did not yield any precipitin line when run against serum Y (Fig. 3). Antigen prepared by rupturing either culture or blood organisms gave a broad and thick precipitin line when tested against serum Y (Fig. 3). Absorption of serum Y with packed culture forms of the Y strain completely removed the antibody which reacted with the circulating antigens (Fig. 4). A human serum from a patient known to be chronically parasitized with *T. cruzi* reacted with plasma of mice acutely infected with both the F1 and Y

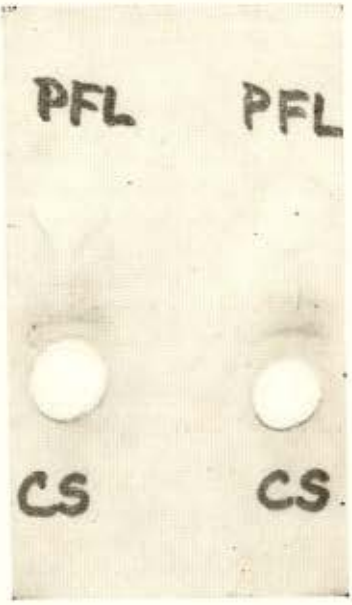

Fig. 2 — CE test. Precipitin lines between wells containing plasma (PFL) of mice acutely infected with *T. cruzi* strain F1 and serum (CS) of mice chronically infected with *T. cruzi* strain Y.

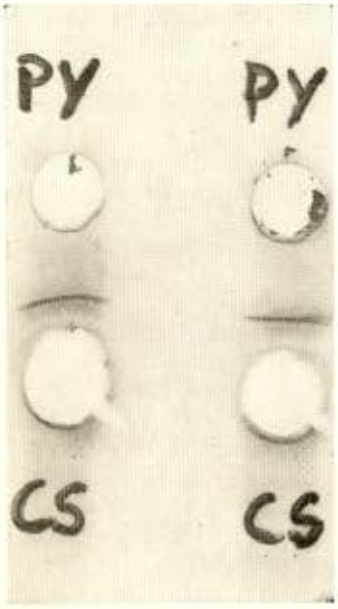

Fig. 1 — CE test. Precipitin lines between wells containing plasma (PY) of mice acutely infected and serum (CS) of mice chronically infected with *T. cruzi* strain Y. In all figures the cathode well is at the top.

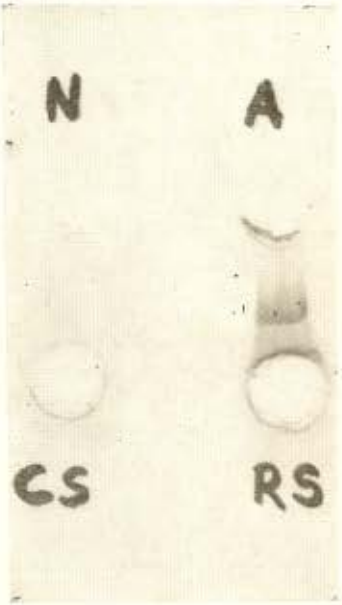

Fig. 3 — CE test. Plasma from normal mice (N) tested against serum of mice chronically infected with *T. cruzi* strain Y (CS). Well A contains antigen prepared from disrupted culture forms of *T. cruzi*. RS = CS.

strains of *T. cruzi* showing a precipitin line which although faint could be easily noted (Fig. 5).

DISCUSSION

The data presented demonstrate that *T. cruzi* releases circulating antigens (exoantigens) into the blood of mice acutely infected with the parasite. The circulating antigens were demonstrated by complement fixation and counterimmunoelectrophoresis. Plasma of acutely infected mice and the supernatant of an ammonium sulphate precipitation of an aliquot of this same plasma both fixed complement in presence of serum from a patient known to be parasitized by *T. cruzi*. The gamma globulins obtained in the precipitation procedure did not fix complement, nor did plasma of uninfected mice. Both substances giving a positive complement fixation reacted at relatively high dilution

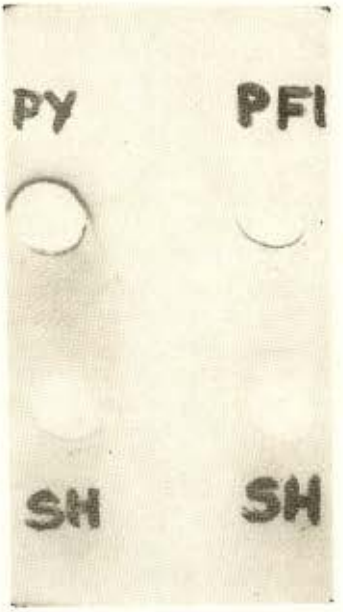

Fig. 5 — CE test — PY and PFL as in Figs. and 2. SH is serum from a human patient know to be chronically infected with *T. cruzi*.

Fig. 4 — CE test. PY as in previous figures. SY = CS as in previous figures. SYA = Serum of mice chronically infected with *T. cruzi* strain Y absorbed with culture forms of the same parasite. Bottom wells filled in the same way as the top wells.

which shows that the concentration of ant gen in them was appreciable. Of intere is the fact that the soluble antigens are pr sent in the supernatant of the precipitati procedure. In African trypanosomes it h been reported that the exoantigens are a sociated to serum proteins other than t gamma globulins [19, 27]. The present resu suggest that this also may be the case f *T. cruzi*. Also the migration towards t anode noted in the CE test agrees with t report of D'ALESSANDRO [8] for exoantige of T. *lewisi*.

Exoantigens either failed to sensitize r cells for the hemagglutination test or t sensitized cells were not able to react wi hemagglutinating antibodies. In some sy tems these antibodies are different from t complement fixing ones, thus it is possil that exoantigens are unable to react wi them although reacting with compleme fixing immunoglobulins.

It has been reported for African tryp nosomes that exoantigens are usually prese in the serum of animals in which there a fulminating parasitemia. However, th relationship is not constant since it may

present in animals with a low parasitemia but absent in animals with a massive infection [10]. In the present report antigen was demonstrated in the blood of animals with a high parasitemia which most probably would result in death of the animals. No antigen was demonstrated in the plasma of mice infected chronically.

ALLSOPP, NJOGU & HUMPHREYS [2] could not detect exoantigens of *T. brucei* in plasma collected right after bleeding the animals. The antigen was demonstrated only after the blood was allowed to stand for at least 15 minutes before centrifugation. They concluded that the exoantigen is not continuously secreted *in vivo* but rather is a surface antigen that is released *in vitro* because of changed environmental conditions. In the present report the antigens were demonstrated in plasma processed as quickly as possible. However, in the centrifuging procedures to prepare plasma free of red cells and trypanosomes at least 40 minutes were spent. All centrifugations were done at 4°C but it is possible that soluble antigens are released even under this condition. In one experiment infected blood was allowed to stand at room temperature for 30 minutes before separating the plasma. The antigen dilution in the CF test and the pattern and density of the precipitin line in the CE test yielded by this plasma was similar to the ones produced by quickly collected plasma. If the findings of ALLSOPP et al. [2] for *T. brucei* were to be applyed to *T. cruzi* one would expect that at least the titer of the antigen in the CF test would be higher since these Authors report increasing amounts of antigen being released while the infected blood is standing unprocessed. The absorption experiments showed that when serum Y was absorbed with packed culture forms the antibody which reacts with antigen present in the plasma was totally removed. This suggests that culture forms have antigens similar to the ones found in plasma.

The pattern and distribution of the precipitin lines between plasma Y and plasma F1 when reacting against serum Y are clearly different.

Parasitemia in mice infected with the F1 strain yields an ascending curve and the predominant trypanosomes are stout although a few slender forms are also present [4]. The 2 lines then may represent antigens released by the 2 morphological forms. On the other hand parasitemia in mice infected with the Y strain shows two peaks, one around the fifth and another around the eight day of infection [4]. It is possible that the 2 peaks of parasitemia may represent antigenic variation. This is widely known to occur in African trypanosomes [15, 16, 22] but so far has not been described in infections with *T. cruzi*.

The functions of the exoantigen have not yet been defined. Rats and mice can be immunized by repeated injection of exoantigens of *T. brucei*, over a period of several weeks. Such mice are protected against infection with the homologous antigenic type of this trypanosomes [19]. Exoantigen may also enhance an infection with trypanosomes when it is added to the trypanosome suspension just prior to inoculation into mice. This effect is proportional to the concentration of exoantigen added [27]. This same property is also suggested for circulating antigens present in serum of rats parasitized with *T. cruzi* [24].

An interesting observation is that serum from a human patient chronically parasitized with *T. cruzi* also yielded a precipitin line when reacting in the CE test with plasma of mice acutely infected with the same parasite. It is possible them that the release of exoantigens also occurs in humans. Or this may be the expression of a similar antigen released by the tissue forms which parasitize infected individuals permanently. Since there is abundant evidence of hypersensitivity phenomena in the pathology of Chagas' disease [21] it is quite possible that soluble antigens may play a role in these allergic manifestations.

Since this paper was written we came across the report by DZBENSKI [11] on exoantigens of *T. cruzi*. This Author also describes the presence of exoantigens in plasma of mice infected with the Sonya strain of *T. cruzi*. The antigens were demonstrated through diffusion in agar, immunoelectrophoresis and immunochromatography. As source of antibody he employed serum of rats and rabbits immunized with plasma of

infected mice. He also reports that the exoantigens have a molecular weight of over 70,000 and contains carbohydrates. Work in progress in our laboratory appears to confirm these findings, however we do not have enough data yet.

RESUMO

Imunologia da Doença de Chagas. I — Antígenos circulantes em camundongos experimentalmente infectados com o T. cruzi

Antígenos circulantes estão presentes em plasma de camundongos com infecção aguda por *T. cruzi*, cepas Y e Fl. Esses antígenos foram demonstrados através das reações de fixação do complemento e imunoeletroforese reversa. São encontrados no plasma total e no sobrenadante de uma precipitação do plasma com sulfato de amônio. Epimastigotas de cultura de *T. cruzi* absorveram totalmente os anticorpos presentes no soro de animais infectados cronicamente com o *T. cruzi*, os quais reagiram contra os antígenos circulantes. Soro de um paciente humano, comprovadamente parasitado pelo *T. cruzi*, também reagiu contra os antígenos circulantes. É provável que tais antígenos estejam presentes em indivíduos com infecção aguda pelo *T. cruzi* e que sejam responsáveis por manifestações de hipersensibilidade descritas na doença de Chagas humana.

ACKNOWLEDGEMENTS

To Dr. Z. Brener for useful discussion and suggestions, and to Dr. J. S. Remington for reviewing the manuscript.

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Recebido para publicação em 11/8/1975.