

**TRYPANOSOMA CRUZI:
CIRCULATING ANTIGENS**

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Circulating antigens were detected in sera of mice experimentally infected with a high dose of Trypanosoma cruzi by reaction with sera from chronically infected mice. The immunodiffusion reaction between homologous acute and chronic sera produced four precipitation lines.

By reaction with chronic mouse serum, circulating antigens were detected in sera from heavily infected hamsters, dogs, rabbits and in sera from chagasic patients. A reaction was also found in urine from acutely infected mice and dogs. Trypanosoma cruzi exoantigen was detected in trypanosome culture medium and in the supernatant of infected cell cultures. Attempts to isolate the antigens are described.

Since the detection of circulating antigens in sera from rats heavily infected with *Trypanosoma brucei* (Weitz, 1960), the importance of such antigens for diagnosis, prognosis, quantification of infection and control of chemotherapy of parasitic diseases has been widely discussed.

The occurrence of circulating antigens in sera of *Trypanosoma cruzi* – infected animals and human patients, and of exoantigen present in trypanosome culture medium has been reported (Tarrant, Fife & Anderson, 1965; Siqueira et al, 1966 and 1979; Dzbéński, 1974; Araújo, 1976; Araújo et al, 1978 and Gottlieb, 1977).

Our work presents some additional facts as primary results in an attempt to isolate and study circulating antigens in Chagas' disease, with the final objective of producing an immunodiagnostic test based on detection of trypanosome – derived material in the circulation of chagasic patients.

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MATERIAL AND METHODS

Trypanosoma cruzi – strains Y (Silva & Nussenzweig, 1953), Brasil (Brand et al., 1949) and Colombiana (Andrade & Andrade, 1966) were used. For experiments with culture forms strains Y and D₁ (isolated in 1932 from a Brazilian chagasic patient and maintained since 1959 by weekly passages on blood agar at the Behring Institute) were used.

Animals – Female 20 g Albany mice (colony breeding, Behring Institute) were used for all experiments unless otherwise described. Inbred mouse strains were obtained from the Laboratory for Animal Breeding and Research Center, GI Bomholtgard Ltd., 8680 Ry, Denmark.

Acute sera – All animals were infected with trypanosomes in citrated or heparinized blood collected from mice at the peak of parasitemia. The animals were injected subcutaneously with the specified infection dose, blood was collected on the peak of parasitemia (day 8 for strains Y and Brasil and day 23 for strain Colombiana); sera separated from clots, centrifuged, filtered through 0.22 μ Millipore membrane and stored at -20°C .

Chronic mouse sera (CMS) was obtained by bleeding mice infected with 0.5 to 10×10^2 blood trypanosomes (via sc) after 80 to 90 days. Sera was obtained as described above and stored in 1 ml aliquots at -20°C .

Rabbit immunization – rabbits were injected subcutaneously with a total of 5 ml acute mouse serum (AMS, Y strain) each in Freund's complete adjuvant, by 5 weekly immunizations. The animals were bled on day 42. Sera were absorbed with normal mouse serum and control of complete absorption of antibodies against normal mouse serum constituents was carried out by counter current immunoelectrophoresis (CIE, carried out according to the Behring technique). After absorption the sera were concentrated by ammonium sulfate precipitation and stored at -20°C .

Immunodiffusion (ID, Behringwerke AG, 1971) and CIE were the methods employed for antigen and antibody detection. For the ID, wells of 6 mm were made, so that 0.1 ml sera aliquots could be used. For the CIE test, the wells had a diameter of 3 mm and 0.02 ml aliquots were used.

For each experiment, pools of sera from 3 mice were used. All experiments were made in reference to normal controls.

Attempts to isolate the antigens included phenol extraction from trypanosome culture medium (Westphal & Jann, 1965) and fractionation of AMS with ethanol according to Cohn (Schultze & Metheka, 1954).

RESULTS

The ID reaction between acute and chronic mouse sera (Brasil strain, Albany mice) produces four precipitation lines (Fig. 1a). The schematic representation (Fig. 1b) shows our arbitrary identification of these lines by numbering them from 1 to 4, starting with the line nearest to the AMS well:

These lines could not be detected if AMS was produced with less than 1×10^6 blood trypanosomes.

To establish the optimal conditions for antigen detection, acutely infected mice (Brasil strain, Albany mice) were bled on days 4, 5, 6, 7 and 8 post infection (p.i.) and tested against CMS. Up to day 5, no antigens could be detected; antigen 3 appeared on

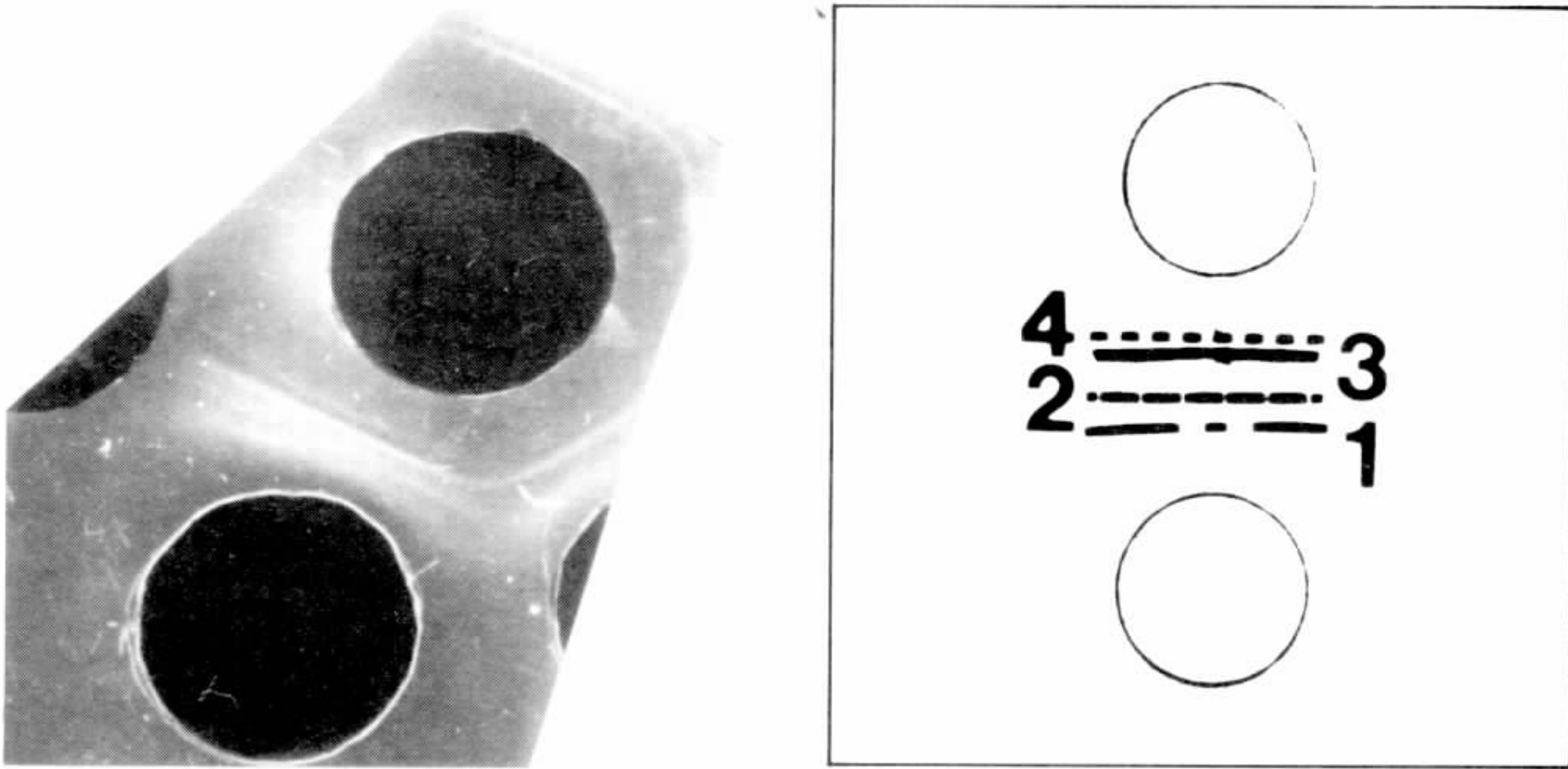


Fig. 1a and Fig. 1b – The upper wells contain CMS, the lower wells AMS.

day 6; antigens 1 and 4 on day 7 and antigen 2 on day 8 p.i. All animals were dead on day 9 p.i. (Fig. 2).

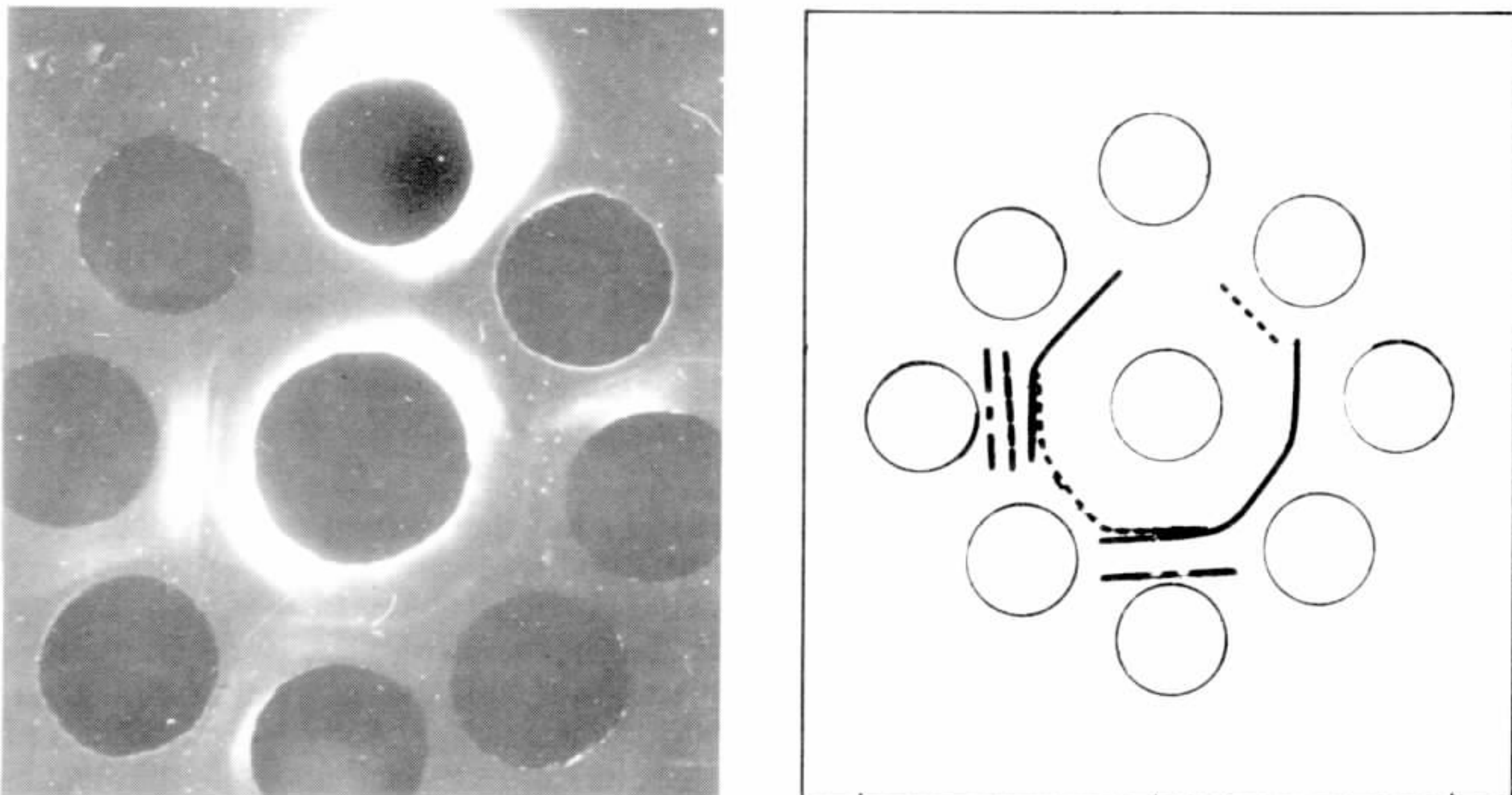


Fig. 2a and Fig. 2b – The central well contains CMS, the outer wells, starting clockwise from the central upper well contain AMS – day 5; trypanosome culture medium; AMS – day 6; sera from acutely infected hamsters; AMS – day 7; trypanosome culture medium; AMS – day 8; and sera from acutely infected hamsters.

The importance of the mouse strain used for AMS production was studied by infecting 9 different mouse strains with 2×10^6 Brasil strain trypanosomes (Table I). The strains Albany and NMRI/WIGA showed identical results for number and kinetics of antigen detection; strains DBA/2/J Bomf, CBA/JCr Bomf and Balb/c Bom-NuNu showed all four antigens but on day 9; and the strains AKR/A Bomf, A/J Bomf, C₅₇BL/6J Bomf and C₃H/Tif Bomf showed different results both in number of antigens detected and in kinetics of antigen occurrence. All animals were dead between days 9 and 10.

TABLE I

Detection (ID against CMS, Brasil strain, Albany mice) of antigens in sera from different mouse strains infected (Brasil strain blood trypanosomes) 4 to 11 days previously.

<i>Mouse strain</i>	<i>day 6</i>	<i>day 7</i>	<i>day 8</i>	<i>day 9</i>	<i>day 10</i>	<i>day 11</i>
Albany	Ag 3	Ag 1, 3, 4	Ag 1, 2, 3, 4	+		
NMRI/WIGA	Ag 3	Ag 1, 3, 4	Ag 1, 2, 3, 4	Ag 1, 2, 3, 4	+	
DBA/2/J Bomf		Ag 3	Ag 1, 3, 4	Ag 1, 2, 3, 4	+	
AKR/A Bomf			Ag 1, 3, 4	+		
A/J Bomf			Ag 1, 3, 4	+		
C ₅₇ BL/6J/Bomf			Ag 1, 3	+		
C ₃ H/Tif Bomf	Ag 3	Ag 2,3	Ag 2, 3, 4	Ag 2, 3, 4	+	
CBA/JCr Bomf	Ag 4	Ag 1, 3, 4	Ag 1, 3, 4	Ag 1, 2, 3, 4	Ag 1, 2, 3, 4	+
Balb/c Bom-NuNu			Ag 2, 3, 4	Ag 1, 2, 3, 4	+	

+ = all animals dead.

Ag = antigen.

The importance of the trypanosome strain used for acute infections was studied by injecting mice with 2×10^6 blood trypanosomes of the Y strain and comparing to mice infected with the same amount of Brasil strain trypanosomes, in the reaction with CMS (Brasil strain). The same precipitation lines were obtained with the two strains in this system (Fig. 3).

To compare the occurrence of circulating antigens in mice infected with blood and culture trypanosomes, mice were infected with 1×10^8 culture forms (D₁ strain). No parasitemia could be observed, and no antigens could be detected on days 2, 3, 7, 8 or 9 p.i. (against CMS – Brasil strain – CIE). Sera obtained from these mice on day 30 p.i. reacted with AMS (Brasil strain), showing only line 1 (Fig. 4).

To test the occurrence of circulating antigens in other animal species, dogs, rabbits, hamsters, rats and pigeons were infected with blood trypanosomes (Brasil strain) and sera from day 8 p.i. tested by ID against CMS (Brasil strain). With an infection dose of 1.4×10^7 trypanosomes per 20g of body weight, hamster sera from day 8 p.i. produced a clear precipitation line, corresponding to line 3 from the homologous system (see Fig. 4). Sera from dogs infected with 3.3×10^4 trypanosomes per 20g of body weight produced occasionally (2 out of 6 infected 4-month-old beagles) a very weak and diffuse precipitation line. Sera from rabbits infected with 1.3×10^5 trypanosomes per 20g of body weight did not react when collected on day 8 p.i., but sera from day 30 p.i. reacted both with CMS and AMS. Sera from rats infected with 7.1×10^6 trypanosomes per 20g of body weight and bled on day 8 p.i. reacted with AMS, and sera from rats bled on day 30 p.i. reacted both with AMS and with sera from rats bled on day 8 p.i. Sera from pigeons infected with 5.4×10^7 trypanosomes per 20g of body weight and bled on days 2, 3, or 8 did not react with either AMS or CMS.

To study the presence of circulating antigens and of antibodies specific for circulating antigens in human chagasic sera, the patient sera available to us (25 sera obtained from Brazilian chagasic patients, no clinical data known) were tested against CMS. Table II shows the results obtained in comparison to hemagglutination (IHA) titers determined using the Behring –Chagas-IHA – kit. Ninety six percent were positive in the

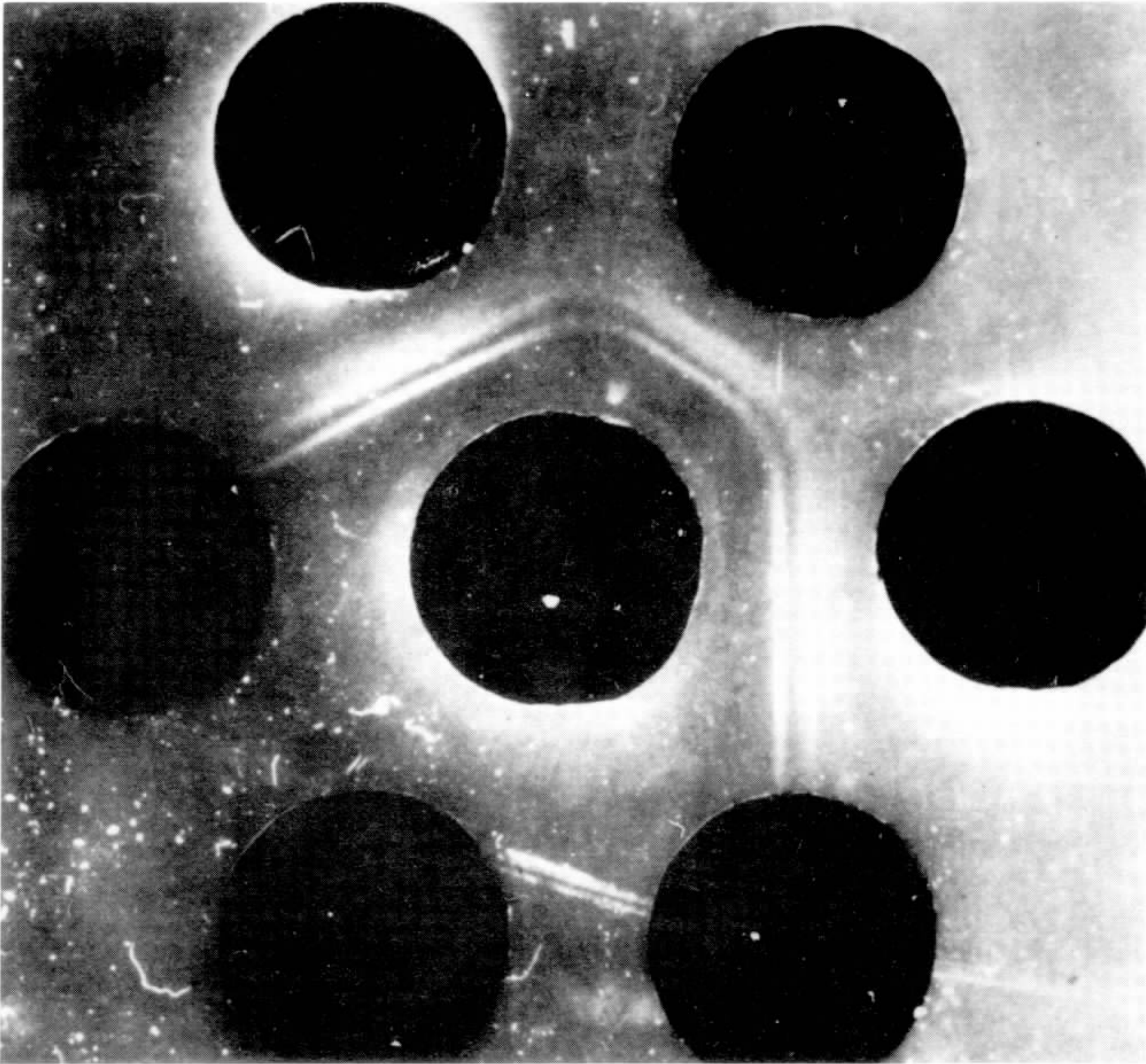


Fig. 3 – The central well contains CMS (Brasil strain); the outer wells, starting clockwise on the higher left well, contain AMS (Brasil strain); AMS (Y strain); and AMS (Brasil strain).

CIE test against AMS; by ID only 56% of these sera were positive. Twenty eight percent were positive in the CIE test against CMS and 24% by ID.

Fig. 5 shows that although precipitation lines were detected, no conclusion can be drawn to identify the lines with the ones formed in the homologous mouse system.

Urine from acutely infected mice (Fig. 6) and dogs (Brasil strain trypanosomes) contain antigen as detected by ID against CMS. Only urine concentrated tenfold by lyophilization of the dialysed material was positive in the ID test.

Exoantigen from *T. cruzi* cultures was detected in trypanosome culture medium. The ID test between sterile filtered overlay from bloodagar cultures, collected on day 5 when trypanosome (strain D₁) concentration was 2 to 3x10⁷ ml, and CMS, showed that the antigen present in the trypanosome culture medium corresponds to the antigen 4 from the homologous mouse system (Fig. 7).

Similar results were obtained with the culture medium of trypanosome infected cells: the centrifuged and sterile filtered medium of Hela or primary mouse heart cells infected 48 hours previously at a 1:10 ratio with blood trypanosomes (Brasil strain) reacted with CMS producing line 4 of the homologous CMS-AMS system.

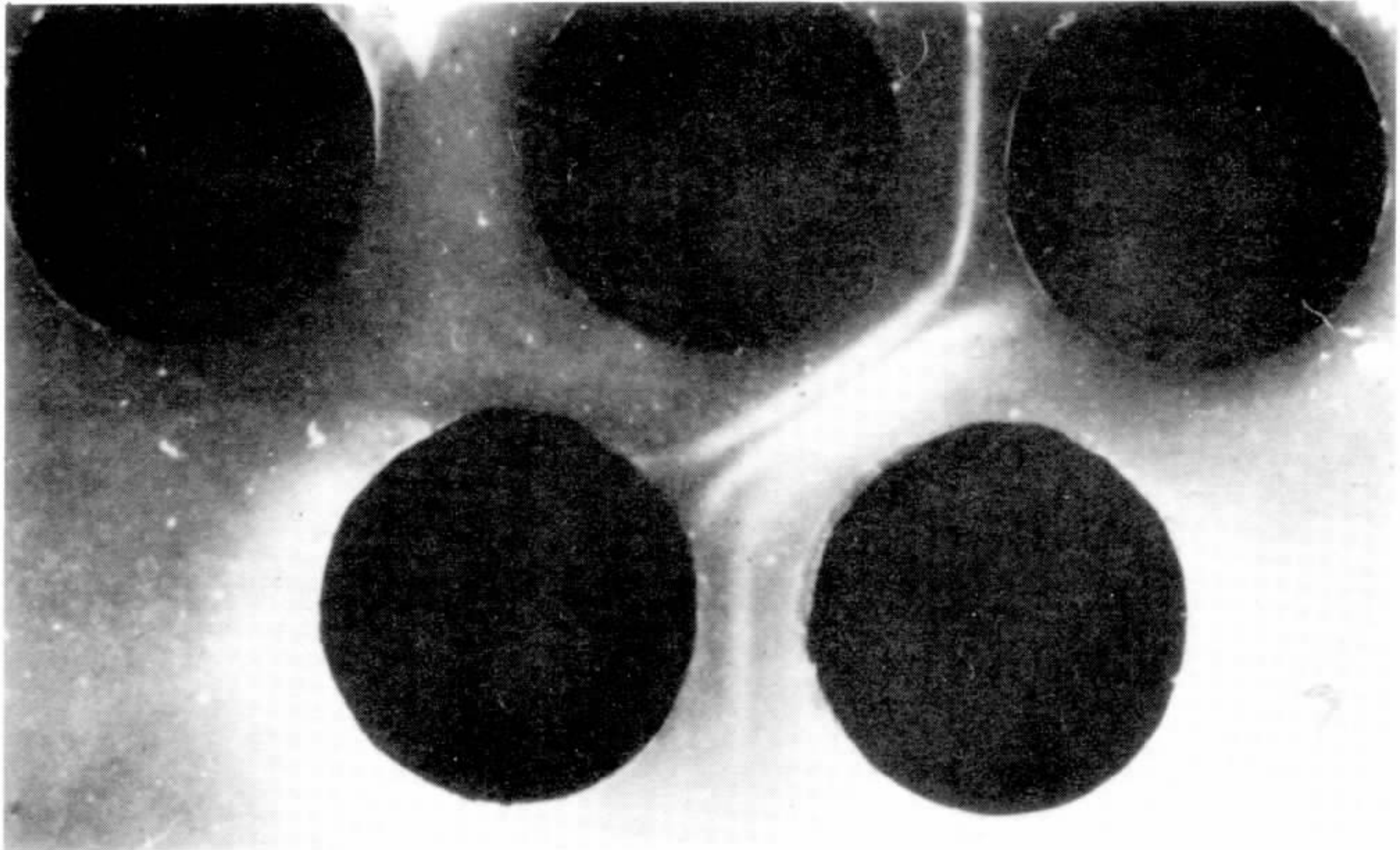


Fig. 4 – The central well contains CMS (Brasil strain); the outer wells, starting clockwise on the right well, contain serum from acutely infected hamsters; AMS (Brasil strain); and serum from D_1 – infected mice (day 30 p.i.).

The crude homogenate or the saline extract from homogenated culture trypanosomes (D_1 , Brasil or Y strains) also react with CMS producing line 4. No other lines could be detected in this system.

Simple extraction of live culture forms with PBS (Guimarães, Ribeiro & Camargo, 1977) also seems to liberate Ag. 4. Line 4 can also be found when live culture forms or isolated (Bongertz & Hungerer; 1978) blood trypanosomes are treated with trypsin (2×10^6 trypanosomes, 1 mg trypsin, 1 ml PBS, 60 min. at 37°C with slow stirring).

Attempts to produce CMS using Brazilian white mice infected with Y or Colombiana strain blood trypanosomes were not as successful as using Brasil strain trypanosomes and Albany mice. Only 2 lines could be detected by ID reaction with AMS (strains Y, Brasil or Colombiana). Comparison with the homologous Brasil strain AMS-CMS system indicate that lines 1 and 3 are present. Therefore, rabbits were immunized with AMS (Y strain), and the antibodies thus obtained (two lines in ID against AMS) used for isolation tests.

The concentrated rabbit globulins were bound to CN Br activated Sepharose 4 B and employed to absorb antigens from AMS (Y strain). Only Ag 3 could be detected in the concentrated fractions obtained after incubation with the dissociation buffer (3 M KBr – glycine buffer, pH 11).

Chemical isolation of the antigens from AMS was attempted, but the methods initially employed (polyethyleneglycol precipitation, gel filtration, ion exchange chromatography and preparative polyvinyl chloride electrophoresis) gave very poor results as evaluated by purification factor and recovery of immunologically active material. Reasonable results were obtained with Cohn's method for plasma fractionation with ethanol: two antigens (seemingly Ag 1 and Ag 3) were obtained in the fibrinogen – containing fraction (Cohn's fraction I, III-3).

TABLE II

Presence of circulating antigens and of antibodies specific for these antigens in human chagasic sera.

Serum no	IHA Titer	CIE		ID	
		Ab	Ag	Ab	Ag
1	256	+	+	+	+
2	512	+	+	+	+
3	512	+	-	+	-
4	512	+	-	-	-
5	1024	+	-	+	-
6	1024	+	-	+	-
7	128	+	+	+	+
8	256	+	-	+	-
9	256	+	-	+	-
10	128	-	-	-	-
11	512	+	+	+	+
12	256	+	-	-	-
13	256	+	-	+	-
14	128	+	-	+	-
15	128	+	+	+	+
16	512	+	-	-	-
17	512	+	+	+	-
18	256	+	+	-	-
19	4	+	-	-	-
20	512	+	-	+	+
21	128	+	-	-	-
22	4096	+	-	-	-
23	4	+	-	-	-
24	256	+	-	-	-
25	128	+	-	-	-

Isolation of Ag 4 from trypanosome culture medium (gel filtration, ion exchange chromatography, preparative polyvinyl chloride electrophoresis, Convanalin A Sepharose absorbtion, trichloroaceticacid precipitation, isoelectric precipitation (Tarrant, Fife & Anderson, 1965) or chloral hydrate extraction (Gonçalves & Yamaha, 1969) was patially successful, but although purification factors were up to 30 (as calculated from sugar concentration determined according to Park & Johnson, 1949, and protein concentration, determined according to Lowry - Bergmayer et al, 1974), the recovery of the immunologically active material (as determined from CIE dilution series) was between 5 and 20% of the initial value.

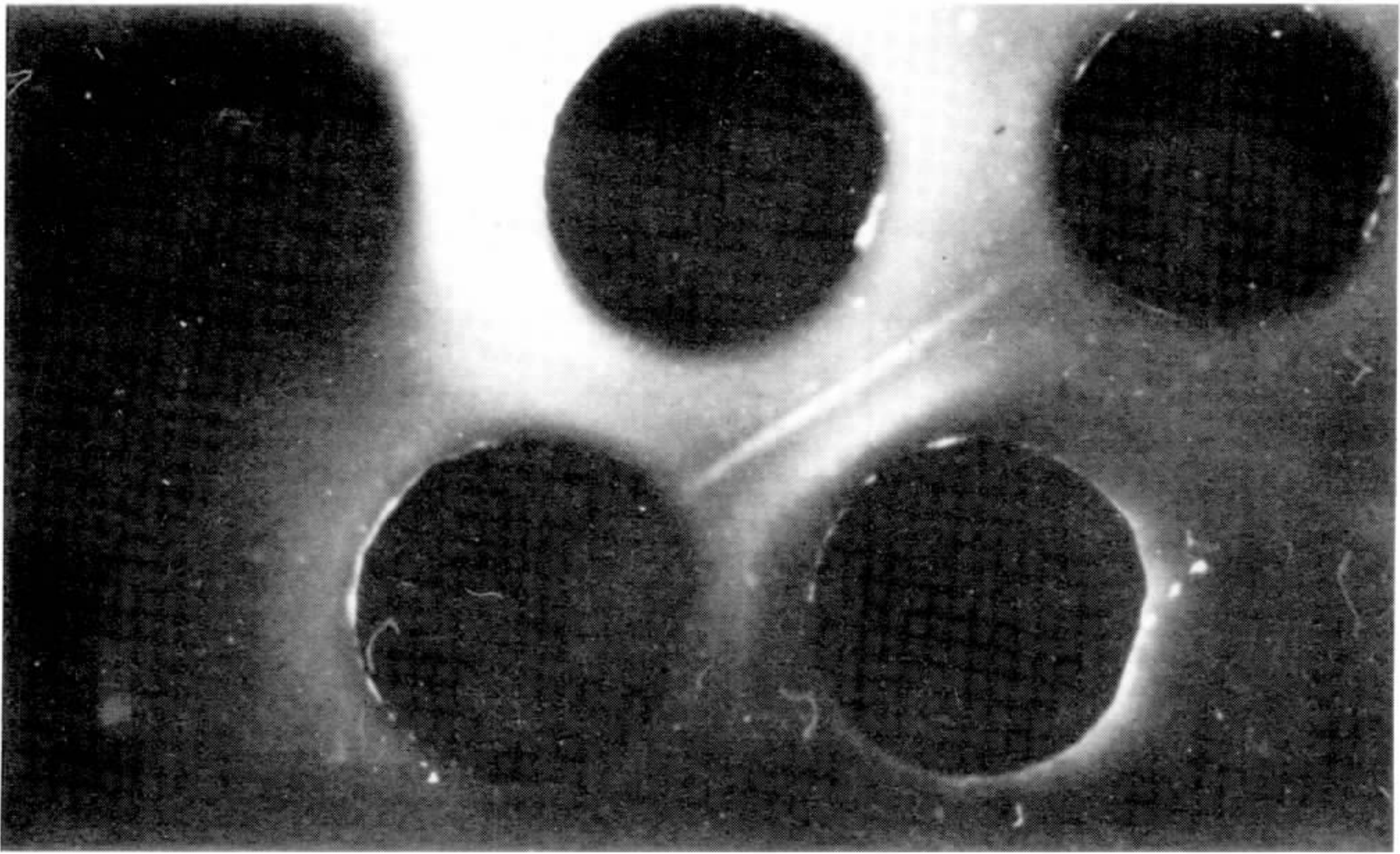


Fig. 5 – The central well contains CMS; the outer wells, starting clockwise on the high right side, contain human serum n^o 14; AMS (Brasil strain); human serum n^o 15; and human serum n^o 16.

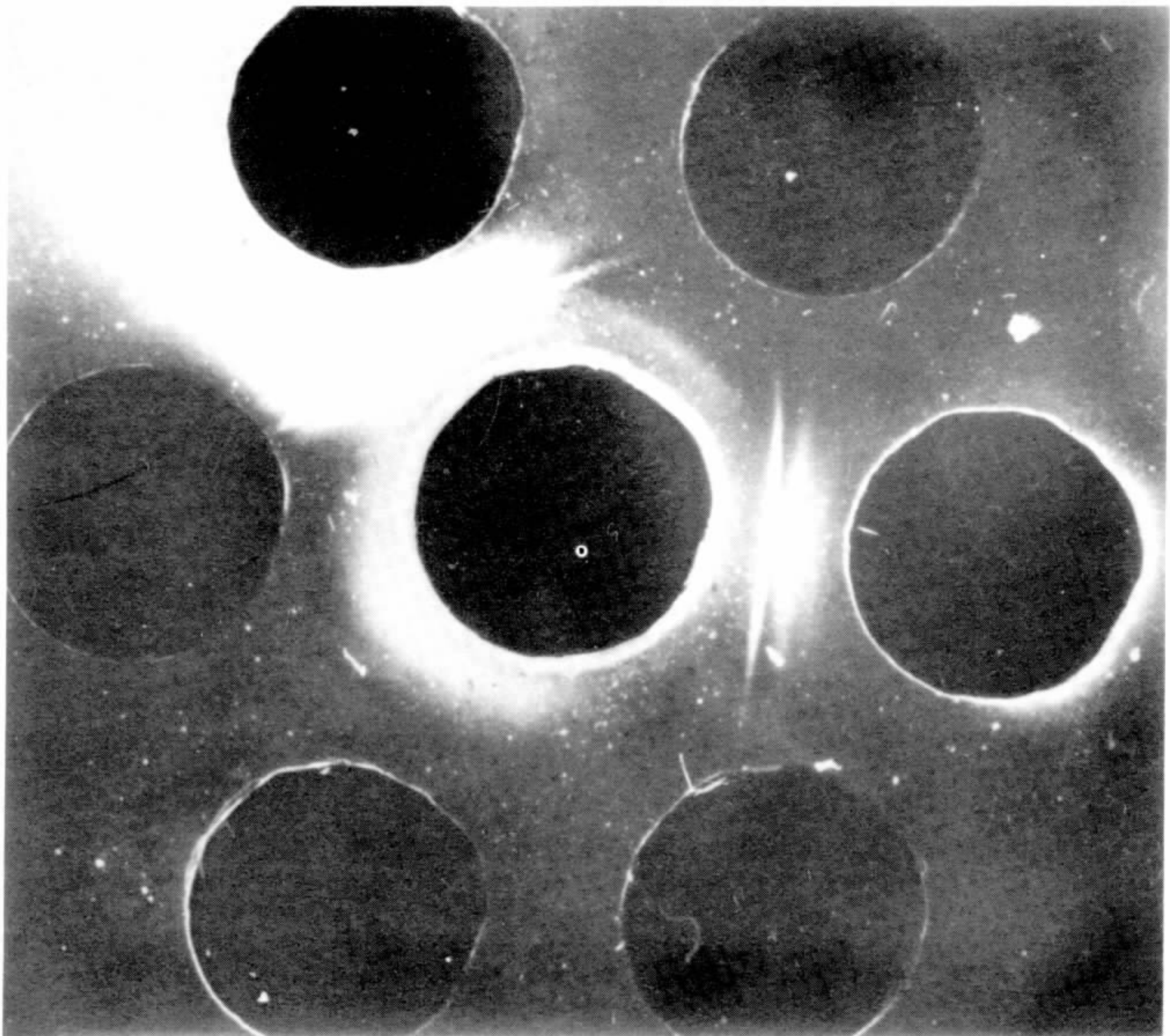


Fig. 6 – The central well contains CMS; the outer wells, starting clockwise on the upper left, contain AMS (Brasil strain); concentrated mouse urine; and AMS (Brasil strain).

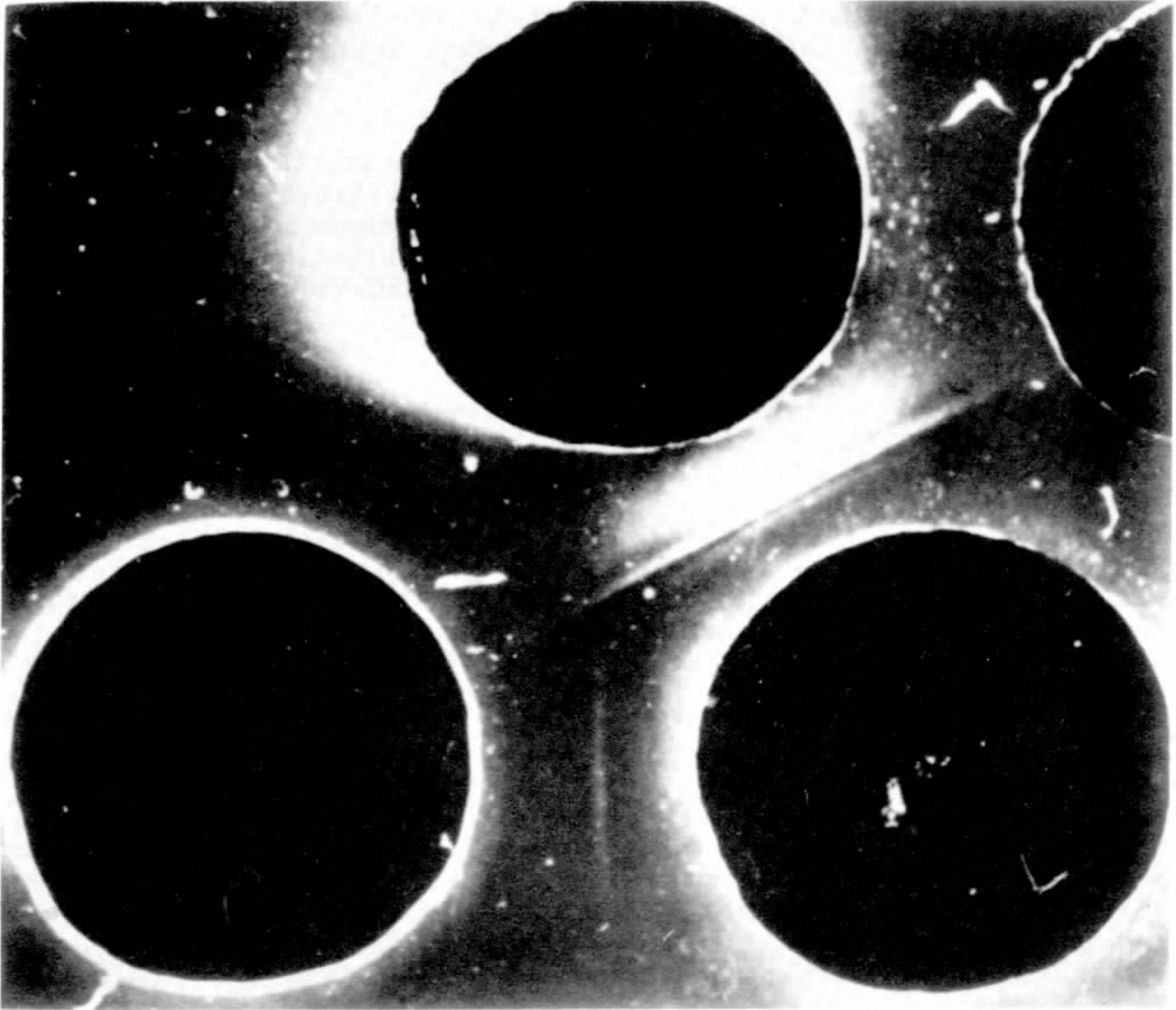


Fig. 7 – The higher well contains AMS, the lower right well contains CMS and the lower left well the 0,22 μ Millipore membrane filtered culture medium.

For isolation of Ag 4 from trypanosome culture medium, the most promising method was phenol extraction (Westphal & Jann, 1965): purification factor = 15, recovery = 100 to 120% (see discussion).

DISCUSSION

Since 1965, experiments have been done on circulating antigens in chagasic infections. Some of the results seem contradictory. Thus, Tarrant et al, 1965, detected an exoantigen in trypanosome culture medium, but Gonzales Cappa et al, 1975, were not able to confirm this result, suggesting that the antigen detected by Tarrant et al is not an exoantigen but a substance derived from the breakdown of trypanosomes. Dzbénski, 1974, reported the presence of an antigen in trypanosome culture medium that did not react with specific antibodies produced in rabbits by immunization with acute mouse serum. Our experiments showed that unconcentrated trypanosome culture medium indeed does not react with antibodies produced according to Dzbénski, but the concentrated phenol extracted antigen does react with these antibodies. We were also able to show that serum of chronically infected mice reacts with trypanosome culture medium producing line 4 of the homologous system in the mouse, thus indicating the presence of one at least antigenically very similar compound both in trypanosome culture medium and in sera of acutely infected mice. Based on this results, we presume that the antigen

not only is a real exoantigen from *Trypanosoma cruzi*, but that it originates from the trypanosome membrane, possibly being the glycoprotein already described by Gottlieb, 1977 and 1978.

Detection of circulating antigens in sera of acutely infected animals or in human sera was reported by reaction with sera of chagasic patients (Araújo, 1976 and 1978; and Siqueira et al, 1966 and 1979); antisera produced by immunization of rabbits with trypanosome homogenates (Siqueira, 1979) or isolated antigen (Gottlieb, 1977), or sera of acutely infected animals (Dzbeniski, 1974); and by reaction with chronic mouse or rat serum (Araújo, 1976).

Only Araújo, 1976, worked in an homologous system (mouse and rat), reporting the presence of two circulating antigens. These results were confirmed by our studies with sera from chronically infected Brazilian white mice. Based on our results, we conclude that the differences in number of antigens in sera from acutely infected mice result from the production of CMS using different mouse strains: using Y strain AMS produced in Albany mice against CMS produced in Albany mice with Brasil strain trypanosomes we obtained four precipitation lines; using the same AMS against CMS produced with Y strain trypanosomes in Brazilian outbred mice we obtained only two lines.

These differences in CMS production in different mouse strains do not seem to happen in AMS production. Although different mouse strains tested for antigen occurrence presented slightly different results, these differences seem to be time – dependent and, most probably, also antigen-concentration – dependent. The experiment with the different mouse strains ought to be repeated with larger numbers in each group, so that some animals could survive for more than the 9 days observed with most of the strains. A smaller concentration of a certain antigen would not be detected by the test used throughout this study; the Ouchterlony immunodiffusion test, while being indicated for identification of lines and comparison of antigen and antibody sources, is one of the least sensitive immunological methods.

The study of the human chagasic sera seems to validate our efforts to isolate the antigens to be employed in an immunodiagnostic test. Although only 24 – 28% of the sera studied were positive in reaction with CMS, the high positivity in the CIE test against AMS indicate that although circulating antigens could not be detected in all sera, they are – or have been – present, leading to antibody production.

The isolation of circulating antigens from AMS by Cohn's ethanol serum fractionation, while yielding only very small protein concentrations, with fibrinogen as major contaminant, are being continued, as the material obtained seems to be at least antigenically similar to the native substances. This material will be used for antibody production in rabbits and, after absorption with Cohn's fraction I, III-3 from normal mouse serum, should give a higher reaction titer than the antibodies produced by immunization of rabbits with crude AMS.

The phenol extraction from trypanosome culture medium does not produce a pure fraction (as tested by disc-electrophoresis), but the recovery titers obtained were exceptionally high. The material employed for isolation is trypanosome culture medium concentrated by lyophilisation, and it is possible that the phenol extraction "activates" material aggregated during concentration, thus explaining the very high percentage of recovery obtained.

Our objective is to isolate the circulating antigens, study their origin and importance in the pathology of Chagas' disease, produce specific antibodies against the isolated

— or at least enriched — antigens, and determine the use of these antibodies in detection of Chagas' — specific antigen in human patients by means of a more sensitive assay (RIA, ELISA) than the methods employed up to now.

Projects have been outlined to produce monoclonal antibodies against circulating antigens using the hybridoma technique, and are to be started soon, in parallel to our attempts for chemical isolation of the antigens from AMS and from trypanosome culture medium.

RESUMO

Antígenos circulantes foram detectados em soros de camundongos infectados experimentalmente com elevadas doses de *Trypanosoma cruzi* pela reação com soros obtidos de camundongos em fase crônica da infecção. A reação de imunodifusão entre soros homólogos agudo e crônico produziu quatro linhas de precipitação.

Por reação com soro crônico de camundongo, antígenos circulantes foram detectados em soros de cricetos, cães e coelhos infectados com doses elevadas de *Trypanosoma cruzi* e em soros de pacientes chagásicos. Uma reação foi também observada com urina de camundongos e cães infectados de forma aguda. Exoantígeno de *Trypanosoma cruzi* foi detectado em meio de cultura de tripanosomas e em sobrenadantes de culturas de células infectadas. Tentativas de isolamento dos antígenos são descritas.

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