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Isolation of *Trypanosoma cruzi* from Blood by Histopaque and Continuous Percoll Gradient Centrifugations

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ABSTRACT

Separation of the blood forms of trypanosomes from the blood of infected animals is difficult, especially in the case of *Trypanosoma cruzi* Y strain. Two procedures to isolate the Y strain blood forms of *T. cruzi* using polyvinyl pyrrolidone-coated silica (percoll) and histopaque are reported in this study. The recovery rates of parasites were 16±5 and 68±16%, respectively. The parasites isolated by these methods presented normal motility and morphology and were infective to albino mice with prepatent periods, parasitemia curves, and polymorphism patterns during the infection that were similar to those of control parasites. In addition, the preservation of surface antigens was confirmed by immunocytochemical studies.

Index Entries: *Trypanosoma cruzi;* bloodstream trypomastigotes; isolation of *Trypanosoma cruzi.*

INTRODUCTION

Several methods of purifying blood forms of *T. cruzi,* the etiological agent of Chagas disease or American trypansomiasis, have been described.

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However, obtaining blood-stage parasites has presented some difficulties. Budzko and Kierszenbaum (*1*) developed a procedure for isolating and concentrating circulating trypanosomes by differential centrifugation using Ficoll-Hypaque. Nevertheless, the parasites thus obtained were contaminated with leukocytes, platelets, and sometimes erythrocytes. Similar results were obtained by Loures, Pimenta, and Souza (*2*) using metrizamide and by Sanderson, Thomas, and Twomey (*3*) with dextran.

Ion-exchange chromatography using DEAE-cellulose columns was introduced by Lanham (*4*) to separate salivary trypanosomes from the blood of infected rats and mice. This separation depends on the surface charge of many species of trypanosomes being less negative than that of the blood cells of rats and mice at a pH range of 6.0–9.0. However, it was not possible to separate *T. cruzi* Y strain in this way because of the small differences in surface charge between this trypanosome strain and mouse blood cells (*4,5*).

A method of purifying bloodstreams trypomastigotes of several *T. cruzi* strains using DEAE-cellulose columns was standardized by Souza (*6*). This procedure is a modification of Lanham and Godfrey (*5*) method and differs in some aspects from others described to purify *T. cruzi* bloodstream trypomastigotes mainly by avoidance of preliminary purification of parasites. Souza (*6*) used as eluent phosphate-saline-glucose (PSG) at various ionic strengths (*I*). Columns equilibrated with PSG ($I=0.217$) and eluted with this same buffer or columns equilibrated with PSG ($I=0.109$) rising in ionic strength to 0.362 were unsuccessful.

Under such conditions, the parasites, when recovered, were contaminated with erythrocytes. The accepted standard conditions were achieved using columns equilibrated with PSG at $I=0.181$ and consecutive elutions with PSG at $I=0.181$ and $I=0.217$. Although the running of PSG at $I=0.181$ to determine very low or no recovery of parasites, its use is maintained because it allows the subsequent desorption of higher numbers of isolated parasites than when PSG at $I=0.217$ is passed through the columns. The author thus established conditions that could discriminate between the parasite surface charge and those of mouse blood cells. However, this gave low recovery percentages for the *T. cruzi* Y and Berenice strains.

Here we present two procedures for isolating blood Y strain trypomastigotes: continuous percoll gradients and histopaque centrifugations. The Y strain has been characterized as a type I strain (*7,8*) with rapid multiplication rate in the host, maximum parasitemia and mortality from 7–12 d of infection, and predominance of slender forms and macrophagotropism in the early phase of infection. The motility and morphology of the isolated parasites as well as their biological characteristics and antigenicity were evaluated.

METHODS

Parasites

The *T. cruzi* Y strain (*9*) was maintained in our laboratory by routine passage in mice.

Infection of Animals

First, 1×10^5 trypomastigotes/mouse were inoculated intraperitoneally into 25 outbred Swiss albino mice weighing 19 ± 4 g. Apoproximately 500 μL of infected blood were collected at peak parasitemia (7th d) from the orbital plexus using heparinized Pasteur pipettes. The infected blood was pooled, and aliquots were diluted in 0.85% ammonium chloride to lyse the erythrocytes (*10*), and parasite numbers were determined using a Neubauer hemocytometer.

Isolation Procedures

Isoosmotic percoll (IOP) was prepared for method A by mixing nine parts of percoll (density 1.130 g/mL, Pharmacia) with one part 2.5*M* sucrose. Dilutions of the IOP in 0.25*M* sucrose were made to obtain 30 and 40% solutions. To form a continuous linear gradient, we used the conventional gradient mixer using 7 mL of each solution. Infected blood (2 mL) diluted with 0.15*M* NaCl in 1:1 (v/v) ratio was layered on top of the gradient. A tube containing gradient solution plus density marker beads (DMB, Pharmacia) instead of blood was run in parallel. Tubes were spun in a swing-out rotor at 400*g* for 40 min at room temperature (RT). After centrifugation, the gradient fractions were harvested with a 50 μL pipet at specific densities as determined from the parallel tube DMB. Each fraction was washed twice in 0.15*M* NaCl.

Isolation of trypomastigotes using histopaque-1.077 (Sigma), method B, was initially carried out according to the method of Chess and Schlossman (*11*) for lymphocyte purification. Nine milliliters of 1:3 dilution in 0.15*M* NaCl of infected blood were layered over 3 mL of histopaque. Tubes were centrifuged at RT for 40 min at 400*g*. Trypomastigotes became concentrated in a ring together with lymphocytes. To further purify trypomastigotes, these rings were harvested, diluted in 0.15*M* NaCl, and centrifuged at 400*g* for 10 min to sediment the lymphocytes. The tubes were then kept at RT for 30 min to swim out of the parasites from the pellet. The supernatant containing trypomastigotes was collected and centrifuged at 700*g* for 10 min. The supernatant was discarded, and the pellet (trypomastigotes) was washed twice in 0.15*M* NaCl.

The percentages of recovered trypomastigotes and purity were determined following both procedures. The motility of the parasites isolated was observed in fresh preparations, and their morphology was verified by examining Giemsa stained smears.

Biological Characteristics of Isolated Trypomastigotes

Outbred Swiss albino mice were infected to determine the infectivity, prepatent periods, and parasitemia curves of isolated trypomastigotes. Groups of 10 mice were inoculated intraperitoneally with 1×10^5 trypomastigotes from fractions isolated by both procedures. Controls consisted of trypomstigotes isolated by differential centrifugation according to the method of Teixeira and Yoshida (*12*). Parasitemia and morphology of bloodstream parasites during infection were evaluated according to Brener (*13*) and Andrade (*7*). Infectivity was evaluated by evidence of parasite multiplication, and virulence was estimated by intensity of this multiplication according to the method of Andrade (*7,8*).

Antigenicity of Isolated Trypomastigotes

An enzyme-labeled antibody method (ELAM) (*14*) was carried out to verify whether trypomastigotes isolated by both procedures had preserved their antigenic composition. Formalin-fixed parasites were smeared onto slides and air-dried for 30 min at 37°C The slides were incubated with 3% milk in PBS pH 7.2 for 30 min to block nonspecific binding sites. After washing, the slides were incubated at RT for 30 min with 50 μL of pooled serum from infected mice or for controls, with pooled serum from healthy mice. Following three washes with PBS, slides were incubated with 50 μL of 1:100 rabbit antimouse horseradish peroxidase-labeled immunoglobulin (Ig) (Miles Yeda, Ltd) for 30 min at RT. Slides were then incubated with 0.04% 3,3'-diaminobenzidine (DAB) and 0.02% H_2O_2 in 50 m*M* Tris-HCl (pH 7.6) for 15 min, after which they were washed, dried, and mounted. The intensity of immunochemical staining was evaluated by microscopy by comparing with control slides using normal mouse serum instead of anti-*T. cruzi* serum.

RESULTS

Isolation of Parasites

The recovery rate of parasites isolated by continuous percoll gradient was 16±5% located between 1.054 and 1.067 densities. The continuous gradient and the corresponding DMB control in shown in Fig. 1. The con-

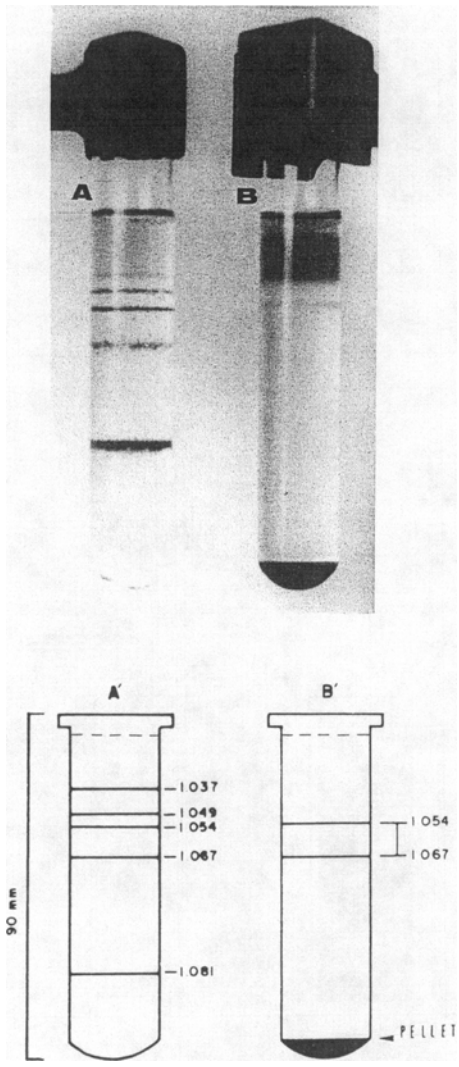

Fig. 1. Purification of bloodstream trypomastigotes by continuous Percoll gradient. **A:** layering of density marker beads. **B:** experimental continuous gradient. **A'** and **B':** schematic representation of A and B, respectively.

centration of trypomastigotes obtained by histopaque is illustrated in Fig. 2. After centrifugation, a white ring containing trypomastigotes, lymphocytes, and platelets was seen in the tube. The recovery of parasites after centrifugation of the ring fraction was $68 \pm 16\%$. In both procedures, contamination by erythrocytes and lymphocytes, in 10 μL of cell suspension, as stained by Giemsa (Fig. 3) was absent, but platelets remained. One hundred percent of these isolated by both procedures presented normal motility; they also presented well preserved morphology as evidenced in Giemsa stained preparations (Fig. 3).

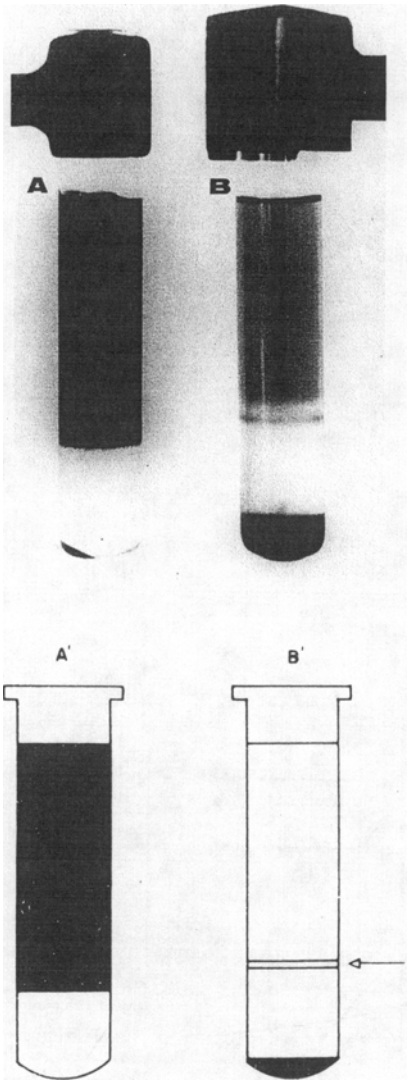

Fig. 2. Purification of bloodstream trypomastigotes using Histopaque. **A:** diluted blood above the histopaque. **B:** ring containing the parasites, lymphocytes, and platelets (*see arrow*). **A'** and **B'**: schematic representation of A and B, respectively. Erythrocytes and granulocytes were found at the bottom on the tube.

Biological Characteristics of Isolated Trypomastigotes

There was no difference in the course of infection as shown by prepatent periods and parasitemia peaks (Fig. 4) between mice infected with isolated trypanosomes and controls. Virulence also remained unchanged, since the cumulative mortality of the mice was 100% at 13 d after infection, as was the control group. Slender forms were predominant during infection (Fig. 5).

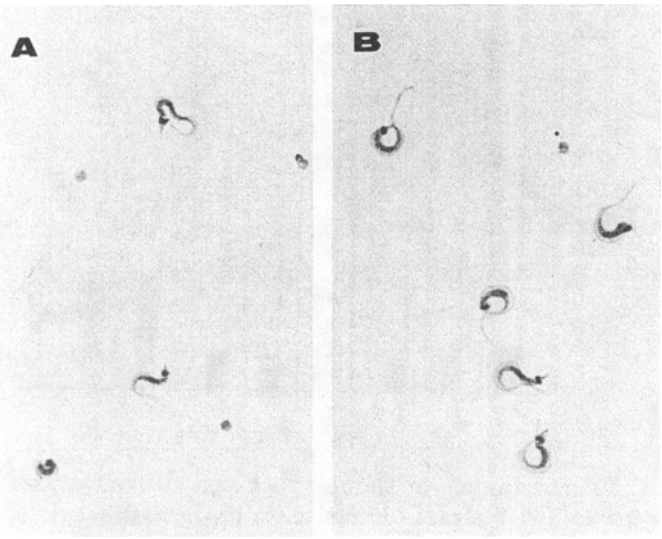

Fig. 3. Bloodstream trypomastigotes of *T. cruzi* Y strain isolated by continuous Percoll gradient (**A**) and Histopaque (**B**).

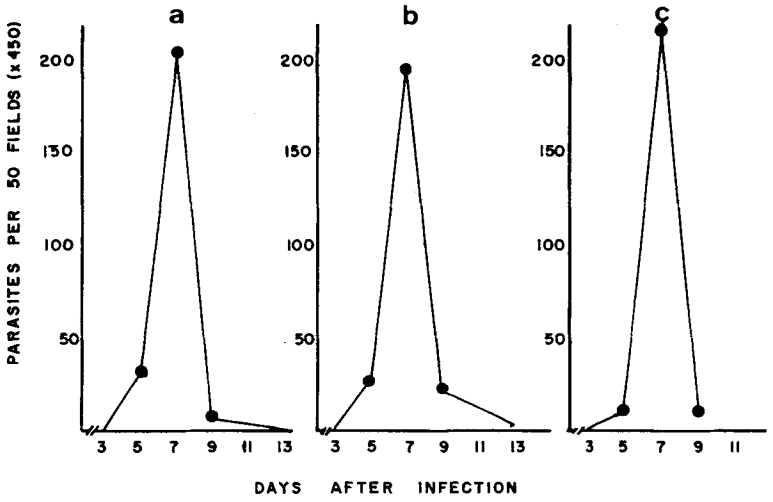

Fig. 4. Course of parasitemia in mice inoculated intraperitoneally with 1×10^5 bloodstream trypomastigotes. **A:** isolated by Percoll. **B:** isolated by Histopaque. **C:** control.

Antigenicity of Isolated Parasites

By using ELAM, serum from infected mice showed intense color reactions against isolated trypomastigotes (Fig. 6). The reaction was not observed in the control by using serum from normal mice (Fig. 6).

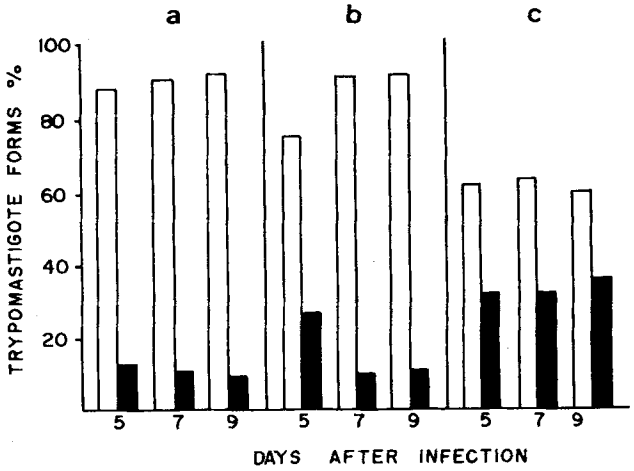

Fig. 5. Predominance of slender forms in the peripheral blood of mice inoculated with 1×10^5 Y strain bloodstream trypomastigotes. **A:** parasites isolated by Percoll. **B:** parasites isolated by Histopaque. **C:** control. □ slender forms; ■ broad forms.

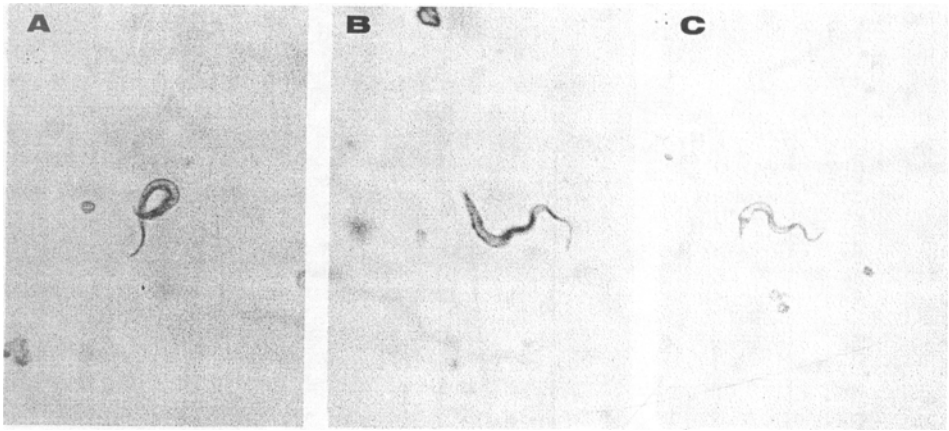

Fig. 6. ELAM reaction of *T. cruzi* bloodstream trypomastigotes Y strain isolated by Percoll (**A**) and by Histopaque (**B**). The reaction is not observed in the control (**C**).

DISCUSSION

This paper describes two methods for isolating *T. cruzi* Y strain bloodstream trypomastigotes using the inert and low-osmotic substances percoll and histopaque. Percoll is composed of colloidal silica coated with polyvinyl pyrrolidone and has been used in the isolation of parasites (*15–18*) and cells (*19*). Histopaque is a premixed commercial preparation, containing Ficoll 400 and sodium diatrizoate ($d = 1.077$ g/cm^3), and it has

been used for lymphocyte isolation (*11*). Previous attempts to purify *T. cruzi* trypomastigotes from blood and cultures were performed using fresh serum (*20,21*). However, the utilization of animal sera presents serious disadvantages when biological, biochemical, or immunological studies are to be performed (*17*).

Another method used for purifying bloodstream trypomastigotes is DEAE-cellulose column chromatography. This method, introduced by Lanham (*4*) to purify salivary trypanosomes and modified by Souza (*6*) for purifying many strains of *T. cruzi* bloodstream trypomastigotes, has not been efficient in obtaining Y strain trypomastigotes. In addition, there were some references to alterations in the membrane of *T. cruzi* that were shown by negative immunofluorescence reaction (*22*) and changes in surface electrical charges as indicated by the cell microelectrophoresis test (*23*). These membrane alterations may have arisen for reasons similar to those of Goldberg et al. (*24*), whereby parasites were filtered through a slurry of DEAE-cellulose packed in Buchner funnels using a vacuum system.

In this work, when continuous percoll gradients were used, bloodstream trypomastigotes were found in densities between 1.054 and 1.067. The total recovery of parasites was $16 \pm 5\%$. These results suggest that *T. cruzi* Y strain is a heterogeneous population. The low density presented by the parasite justifies the high recovery rate when histopaque was used, because this substance has a specific gravity of 1.077. In both procedures, contamination by erythrocytes and leucocytes was absent. However, contamination with platelets was observed. Trypomastigotes isolated by both procedures remained infective in mice; and prepatent periods, parasitemia pattern, and mortality were similar to those of mice infected with control trypomastigotes.

The evaluation of the percentage of broad and slender forms of trypomastigotes in peripheral blood of infected mice has shown that different strains may be characterized by morphologic aspects (*15,25*). Slender forms predominate in highly virulent strains (*26*). Trypomastigotes isolated by both procedures show predominance of slender forms in peripheral blood during the infection, demonstrating that the trypomastigotes preserved characteristics of their virulence. The reactivity of the positive serum when compared with control, by the ELAM method, indicated that parasites isolated by our methods preserved their surface antigens, allowing their use in immunological studies.

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REFERENCES

1. Budzko, D. B. and Kierszenbaum, F. (1974), *J. Parasitol.* **60,** 1037, 1038.
2. Loures, M. A., Pimenta, P. P. F., and Souza, W. (1980), *J. Parasitaol.* **66,** 1058, 1059.
3. Sanderson, C. J., Thomas, J. A., and Towmey, C. E. (1980), *J. Parasitol.* **80,** 153–162.
4. Lanham, S. M. (1968), *Nature* **218,** 1273, 1274.
5. Lanham, S. M. and Godfrey, D. G. (1970), *Exp. Parasitol.* **28,** 521–534.
6. Souza, S. A. (1983), *Mem. Inst. Oswaldo Cruz* **78,** 317–333.
7. Andrade, S. G. (1974), *Rev. Pat. Trop.* **3,** 65–121.
8. Andrade, S. G. (1985), *Rev. Soc. Bras. Med. Trop.* (Suppl.) **18,** 39–46.
9. Silva, L. P. H. and Nussenzweig, V. (1953), *Folia Clin. Biol.* **20,** 191–207.
10. Hoff, R. (1974), *J. Parasitol* **60,** 527, 528.
11. Chess, L. and Schlossman, S. F. (1980), *Clinical Immunology,* Rose, N. R. and Friedan, eds., American Society for Microbiology, Washington, DC, pp. 234, 235.
12. Teixeira, M. M. G. and Yoshida, N. (1986), *Mol. Biochem. Parasitol.* **18,** 271–286.
13. Brener, Z. (1969), *Ann. Trop. Med. Parasitol.* **63,** 215–222.
14. Tachibana, H. and Kaneda, Y. (1984), *Jpn. J. Parasitol.* **33,** 457–466.
15. Hart, D. T., Vickerman, K., and Coombs, G. H. (1981), *Parasitology* **82,** 345–355.
16. Grab, D. J. and Bwayo, J. J. (1982), *Acta Trop.* **39,** 363–366.
17. Castanys, S., Osuna, A., Gamarro, F., and Ruiz-Perez, L. M. (1984), *Z. Parasit.* **70,** 443–449.
18. Chandrashekar, R., Rao, U. R., Rajasekariah, G. R., and Subrahmanyam, R. (1984), *J. Helmintol.* **58,** 69, 70.
19. Ravnik, S. E., Gage, S., and Pollack, S. B. (1988), *J. Immunol. Meth.* **110,** 161–168.
20. Nogueira, N., Bianco, C., and Cohn, Z. (1975), *J. Exp. Med.* **142,** 224–229.
21. Kimura, E., Lay, W. E., and Fernandes, J. F. (1978), *Rev. Inst. Med. Trop.* **20,** 133–138.
22. Souza, W., Chiari, E., Romanha, A. J., and Chiari, C. A. (1976), III Reuniao Anual Sobre Pesquisa Basica em Doenca de Chagas, pp. 12, 13.
23. Souza, W., Arguello, C., Martinez-Palomo, A., Trissl, D., Gonzalez-Robles, A., and Chiari, E. (1977), *J. Protozool.* **24,** 411–415.
24. Goldberg, S. S., Pereira, A. S. S., Chiari, E., Mares-Guia, M., and Gazzinelli, G. (1976), *J. Protozool.* **23,** 179–186.
25. Brener, Z. and Chiari, E. (1963), *Rev. Inst. Med. Trop.* **5,** 220–224.
26. Brener, Z. (1969), *Ann. Trop. Med. Parasitol.* **63,** 215–220.