A glass wool-based method for purifying *Trypanosoma cruzi* trypomastigotes and identification of an epimastigote-specific glass-adherent surface peptide

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Glass wool, hydrophilic cotton wool, non-electrically charged BIO-GEL P2 and common tissue paper columns were used to purify trypomastigotes from a mixed *Trypanosoma cruzi* population grown in axenic culture medium. With all these columns, highly purified (up to 98%) trypomastigote preparations were obtained. Trypomastigote yields from cotton wool, BIO-GEL P2 and common tissue paper columns were not as high as from glass wool columns, from which yields varied from 69 to 80%. Purification on glass wool did not affect trypomastigote infectivity or virulence. Dead trypomastigotes could not be purified on glass wool columns. A glass-adherent amphiphilic peptide of 45 kDa, present in the cell membrane, was isolated from epimastigote but not from trypomastigote preparations.

Introduction

Purified infective forms of *Trypanosoma cruzi* (trypomastigotes) are often needed by investigators studying the parasites, either in vivo or in vitro. This has been achieved in the past by treating *T. cruzi* grown in LIT (liver infusion-tryptose medium, Camargo, 1964) with fresh serum (Nogueira et al., 1975), which lyses only epimastigotes, and not trypomastigotes (Muniz and Borriello, 1945) and then removing the lysed epimastigotes by different techniques (Nogueira and Cohn, 1978; Castanys et al., 1984). A relatively simple procedure, chromatography on DEAE-cellulose, has also been used to purify trypomastigotes from total *T. cruzi* populations obtained from different sources (Al-Abbassy et al., 1972; Goldberg et al., 1976; Alvarenga and Brener, 1979). However, since the surface of trypomastigotes is more negatively charged than that of epimastigotes (De Souza et al., 1977), the fact that trypomasti-

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gotes are eluted from the positively charged DEAE-cellulose column before epimastigotes cannot be explained entirely on the basis of charge interactions.

This, as yet unexplained, relatively high affinity of epimastigotes for DEAE-cellulose, as well as reports of the binding of epimastigotes to the intestinal lining of the insect vector (Böker and Schaub, 1984; Zeledon et al., 1984) and to the walls of glass culture tubes, have motivated the present study on the relative adherence of epimastigotes and trypomastigotes to glass wool, BIO-GEL P2, hydrophilic cotton wool and common cellulose tissue paper.

Materials and Methods

T. cruzi strain   Trypomastigotes and epimastigotes from *T. cruzi* of the Y (Silva and Nussenzweig, 1953) and F (Deane and Kloetzel, 1974) strains were obtained from 10 to 12 day old LIT (Difco Laboratories, Detroit, MI) cultures under conditions optimized for the production of maximum numbers of trypomastigotes. Briefly, in vitro cultures were initiated by adding about 1 ml of blood, taken from infected mice at peak parasitaemia, to 6 ml of LIT medium. After 20–30 days, aliquots of these epimastigote-rich cultures, containing about 10^7 parasites, were added to 6 ml of fresh LIT medium and kept in culture for one week. From these cultures, weekly passages to fresh LIT were performed by inoculating $1–2 \times 10^7$ ml^{-1} parasites into 60 ml of medium in 1000-ml culture Roux bottles. These cultures yielded 50–70% trypomastigotes after 10–12 days at 28°C. Only parasites which had been subjected to a maximum of 30 passages in the 6-ml system were used since cultures of parasites which had been maintained in vitro for longer periods of time contained significantly higher percentages of epimastigotes.

Urine from infected *Triatoma infestans* containing Colombian strain (Federici et al., 1964) metacyclic trypomastigotes and epimastigotes were obtained as described by Zeledon et al. (1977).

Investigation on the binding of parasites to glass   The binding of parasites to glass was investigated by adding small amounts of previously treated glass wool (Pirex Brand, Owens-Corning Fibreglass Corporation, Corning, NY) to LIT cultures (undiluted or diluted 1 : 10 with 0.15 M phosphate-buffered saline (PBS), pH 7.2) with epimastigote/trypomastigote ratios of about 1. The glass wool was treated with sulphochromic solution for 2 h and thoroughly washed with distilled water and PBS prior to use. After 10, 30, 60 and 180 min of incubation at room temperature, the pieces of wool were removed from the cultures and examined by light microscopy.

Purification of parasites   Glass wool, treated with sulphochromic solution and washed as described above, was cut into small pieces with a pair of scissors and firmly packed into 10-ml glass syringes using a glass rod, then washed under suction with PBS. Columns were equilibrated with PBS containing 1% glucose (PBS-glucose).

T. cruzi cultures were harvested by centrifugation ($1000 \times g$, 20 min, 4°C) and the pellets were resuspended with a Pasteur pipet in a minimal volume of culture supernatant. These suspensions were applied to columns packed with various volumes of glass wool (as specified in Table 1 and in legends to figures). Flow rates

were adjusted to 8 ml h^{-1} (unless otherwise stated in the text) and the parasites were eluted with PBS-glucose at room temperature and collected in 1-ml fractions. On one occasion, 10% (final concentration) bovine serum albumine (BSA, fraction V, Sigma, St. Louis, MO) was added to the parasite suspension and to the elution and equilibration buffers. Trypomastigote/total parasite ratios were determined by microscopic examination of live and Giemsa-stained parasites.

Purification on DEAE-cellulose was performed as described elsewhere (Sousa, 1983) in the presence or absence of 10% BSA. The procedures for purification of parasites on BIO-GEL P2 (Bio-Rad Laboratories, Richmond, CA), tissue paper and cotton wool columns were identical to those employed with glass wool columns. The controls and post-column parasites were washed and maintained in PBS with 1% BSA or normal mouse serum for subsequent experiments.

Killing of parasites In order to investigate whether the separation of parasites on the column was due to an active process, parasites were killed by incubation with merthiolate (Eli Lilly, USA) 0.1 mg ml^{-1} in PBS for 10 min, centrifuged and washed twice with PBS, before loading on to the glass wool columns.

Experimental infection Two groups of 10 male albino mice, weighing 11 g each, were injected i.p. with 10^5 pre- or post-glass wool column Y strain parasites. Both parasite preparations were treated with human complement according to Muniz and Borriello (1945) in order to kill epimastigotes. To determine the parasitaemia, samples of blood (5 µl collected from the tail) were dispersed on to a glass coverslip (22 × 22 mm), and the parasites counted using a microscope with 400 × magnification.

Surface iodination One hundred million live Y strain epimastigotes and trypomastigotes were washed three times with 0.15 M PBS and RPMI 1640 medium respectively. The surface proteins were labelled with ^{125}I (NaI, Amersham, U.K.) by using Iodogen (Pierce Chemical, U.S.A.) as the oxidizing agent (Markwell and Fox, 1978).

Purification of glass-adherent peptides Amphiphilic surface proteins were partially purified from radiolabelled epimastigotes or trypomastigotes using Triton X-114 (TX-114) (Bordier, 1981). After phase-separation, the detergent phase was mixed with a solution of 10% Triton X-100 (v : v), and the detergent concentration adjusted to 0.05% by dilution with 10 mM Tris·HCl, pH 7.4. The material was then applied onto a 0.6-ml packed glass wool micro-column made from a 1000 µl automatic pipette tip. After 30 min incubation at room temperature, the micro-column was washed three times with PBS by centrifugation (1000 × g, 10 min). The molecules adsorbed to the glass wool were eluted with 100 µl of 2% sodium dodecyl sulphate (SDS) in PBS, followed by centrifugation as above.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) Glass-adherent proteins were concentrated by precipitation with cold acetone and subjected to SDS-PAGE (Laemmli, 1970). After electrophoresis, the gel was fixed, dried and autoradiographed using an intensifying screen (Eastman Kodak, Rochester, NY).

Results

Binding of parasites to glass wool When glass wool was incubated with undiluted LIT cultures containing about 50% trypomastigotes and 50% epimastigotes, few parasites were found adsorbed to the glass fibres, even if the parasites were previously subjected to centrifugation ($1000 \times g$ for 20 min at 4°C). On the other hand, when the cultures were diluted 1 : 10 with PBS, 95–100% of the parasites that were found adhered to glass after 10 min were epimastigotes (Fig. 1c).

Purification of trypomastigotes on glass wool The yield of LIT-derived trypomastigotes from glass wool columns varied from 68–80% of the total number of applied trypomastigotes (Table 1). The percentage of trypomastigotes in the parasite populations eluting in the first 2.5 column volumes ranged from 92–98% (Table 1 and Fig. 1a). In the subsequent fractions, increasing contamination with epimastigotes was found (not shown).

Metacyclic trypomastigotes obtained from the insect vector could also be purified on glass wool columns. For instance, 250 μl of *Triatominae* urine containing 1.5×10^6 parasites (85% trypomastigotes) yielded a trypomastigote preparation free of epimastigotes (in the first 2.5 ml of effluent), when applied to a 1-ml column, with a recovery of 80% of the trypomastigotes applied. However fecal material, including bacteria, contaminated the purified trypomastigotes.
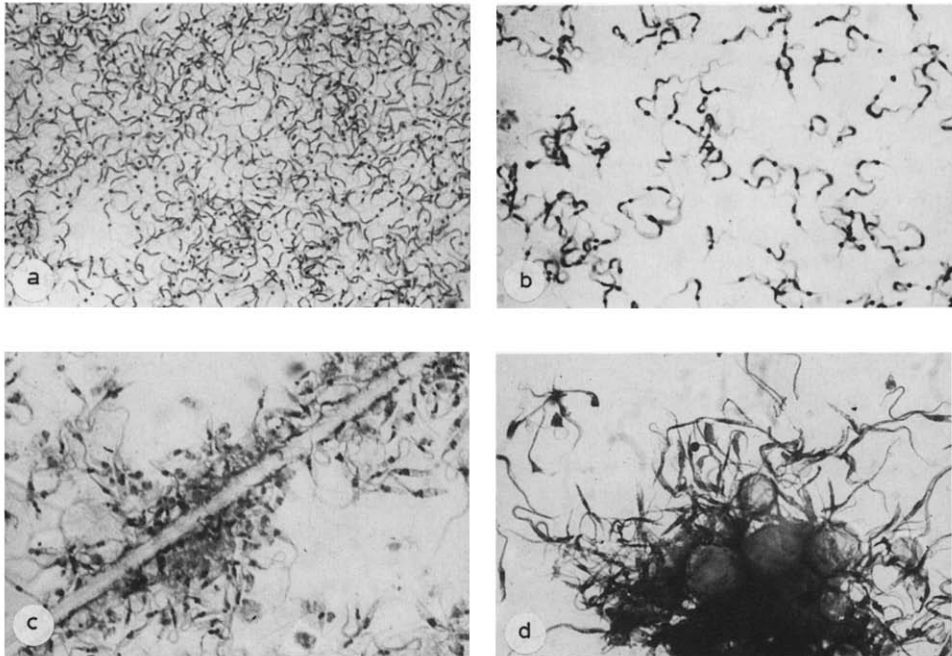

Fig. 1. Purification of Y-strain *T. cruzi* on columns. a, trypomastigotes eluted from a glass wool column; b, trypomastigotes eluted from a tissue paper column; c, parasites retained on material removed from the top of a glass wool column; d, parasites retained on material removed from the top of a BIO-GEL P-2 column. Most of the retained parasites are epimastigotes, which are either bound to glass or BIO-GEL particles or free. a, × 40; b, c and d, × 100; Giemsa stain.

TABLE 1

Purification of trypomastigotes from LIT medium on glass wool and tissue paper column

Column	Column volume (ml)	Volume of applied sample (ml)	Total No. of parasites applied ($\times 10^9$)	Yield of trypomastigotes (%)[a]	Percentage of trypomastigotes in preparations[b]	
					Before purification	After purification
Glass wool	4.5	1.4	2.6	71	50	98
	4	1.3	1.3	69	52	98
	4	1.8	4.6	80	13	98
	3	0.5	0.3	76	50	96
	5	1.6	3.4	70	52	95
	0.6	0.2	0.3	80	57	92
Tissue paper	1.2	0.3	0.01	33	40	97
	3.5	0.5	0.3	50	50	98

[a]% Recovered parasites in relation to applied trypomastigotes.
[b]No. trypomastigotes $\times$ 100/total no. of parasites

Flow rates higher than 8 ml h^{-1} increased the contamination of the preparations with epimastigotes (not shown).

Epimastigotes, as well as a few trypomastigotes, could be seen adsorbed to glass fibers removed from the top of the columns. Some free parasites could also be found between the fibers (Fig. 1c).

Purification of trypomastigotes on BIO-GEL P2, tissue paper and cotton wool Trypomastigotes were preferentially eluted from columns packed with these materials under the conditions described above for glass wool. The yields of trypomastigotes from BIO-GEL (20–30%), tissue paper (Table 1 and Fig. 1b) and cotton wool (15–25%) columns were much lower than that from glass wool columns. Epimastigote clusters bound to BIO-GEL particles, and also free clusters between the particles, were observed in the material removed from the top of the columns (Fig. 1d). BIO-GEL columns were easily blocked when large amounts of parasites were used (i.e. more than 10^9 parasites for a 5-ml column of 1-cm diameter).

Effect of high protein concentration on column purification The presence of 1% or 10% BSA in the equilibration and elution buffers had little effect on the extent to which epimastigotes bound to a glass wool column (Table 2). This contrasted with the observations made with DEAE-cellulose chromatography, namely that a reduction in binding of epimastigotes occurred in the presence of 10% BSA (Table 2).

Effect of killing the parasites on their binding to glass wool The numbers of trypomastigotes eluted from glass wool columns were markedly reduced when they were pre-treated with merthiolate (Table 3). Clusters of dead parasites were found adsorbed to glass fibers when examined by light microscopy.

Biological activity of glass wool purified parasites The possible effect of glass wool fractionation on the biological activity of parasites was investigated by comparing

TABLE 2

Effect of bovine serum albumin (BSA) on the binding of trypomastigotes or epimastigotes to glass-wool and DEAE-cellulose columns

Column material	BSA concentration (%)	% Recovered in relation to applied trypomastigotes	% Recovered in relation to applied epimastigotes
Glass wool	0	68	1.4
	1	73	1.5
	10	71	1.5
DEAE-cellulose	0	76	0.8
	1	74	1.5
	10	79	10.8

TABLE 3

Effect of merthiolate treatment on the purification of *T. cruzi* on glass-wool columns[a]

Treatment	No. of trypomastigotes in the first eluate (2.5 column volumes) ($\times 10^5$)	No. of epimastigotes in the first eluate (2.5 column volumes) ($\times 10^5$)
None	7.8	0.5
Merthiolate[b]	0.2	0.2
Merthiolate[c]	0	0

[a]Sample containing 11×10^5 trypomastigotes and 9×10^5 epimastigotes of Y strain *T. cruzi* were applied to a 3-ml column as described in Materials and Methods.
[b]Parasites were incubated with merthiolate ($10 \mu g \, ml^{-1}$) for 10 min and washed twice with PBS prior to purification.
[c]A second parasite aliquot was treated with merthiolate and washed as described above.

the infectivity and virulence of glass wool purified trypomastigotes with that of parasites not treated with glass wool. No significant differences between the preparations in these two biological parameters could be observed (Fig. 2 and Table 4).

SDS-PAGE of glass-adherent protein SDS-PAGE of surface amphiphilic peptides with affinity for glass, purified from epimastigotes, revealed a marked enrichment of a peptide with M_r of 45 000 (Fig. 3, lane B). No surface peptide with affinity for glass could be detected in the amphiphilic fraction prepared from trypomastigotes (not shown).

Discussion

The present paper shows that glass wool columns are as efficient as DEAE-cellulose columns in the purification of trypomastigotes from total LIT-derived *T. cruzi* preparations. Moreoever, as for DEAE-cellulose chromatography (Gutteridge et al., 1978; Sousa, 1983), the purification on glass wool columns did not affect the infectivity or virulence of the parasites. Glass wool has the obvious advantages of lower
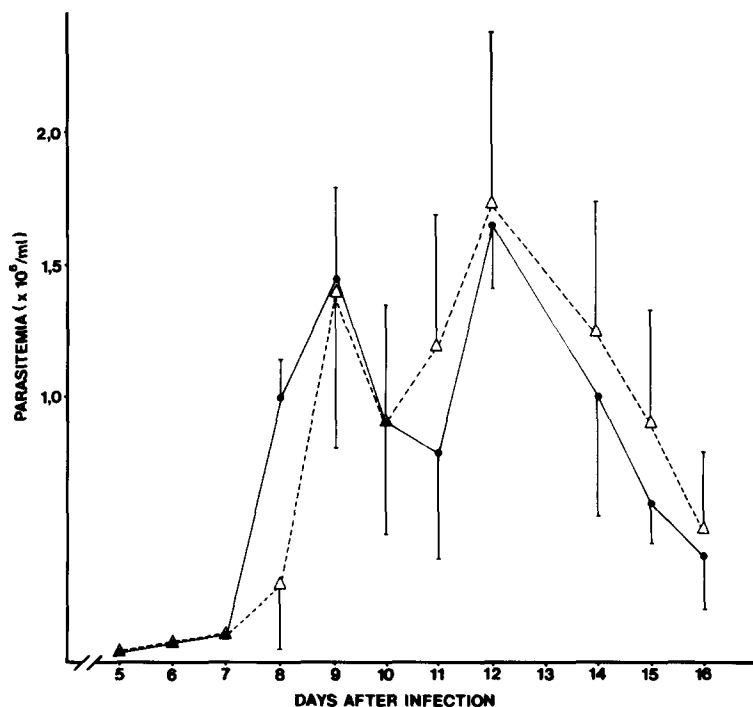
Fig. 2. Parasitaemia curves of mice infected with 10^5 glass-wool purified (●) or non-purified (△) Y-strain trypomastigotes. Each symbol represents the mean number of trypomastigotes per ml of blood from 6 mice; the vertical lines represent the SD.

TABLE 4

Mortality of albino mice infected with 10^5 glass-wool purified or 10^5 non-purified Y-strain trypomastigotes

Trypomastigotes	Days after infection						Cumulative mortality[a]
	18	19	20	21	22	23	
Purified	1[b]	–	1	2	1	1	6/10
Non-purified	–	1	–	2	2	1	6/10

[a]No. of dead mice/total number of mice in group.
[b]No. of dead mice.

costs, higher stability and easier manipulation compared with DEAE-cellulose. Cotton wool, BIO-GEL P2 and common cellulose tissue paper columns were also very efficient in yielding highly purified trypomastigote preparations, but the recovery of trypomastigotes from these columns was lower than that from glass wool columns.

Glass wool columns were also successfully used to separate bug-derived metacyclic trypomastigotes from epimastigotes. However, the contamination by *Triatominae* faecal material, which does not occur in DEAE-cellulose chromatography (Alvarenga and Brener, 1979), constitutes a severe limitation on the use of glass wool columns to purify trypomastigotes from *Triatominae* faeces.

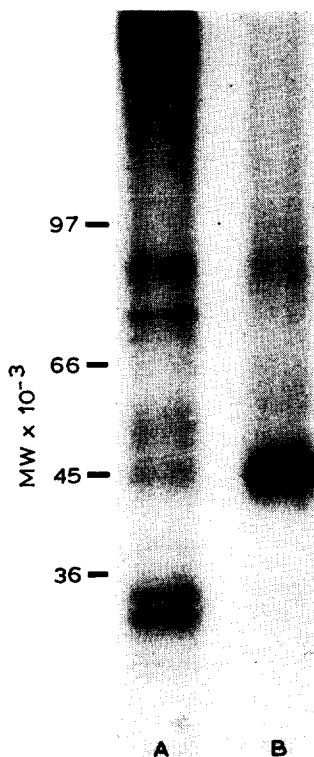Another potential use of glass wool columns would be to purify trypomastigotes

Fig. 3. Autoradiography of SDS-PAGE of extracted TX-114 proteins subjected to glass-wool affinity-chromatography. A, total TX-114 proteins; B, protein eluted from glass wool by 2% SDS. The proteins had been radiolabelled on the surface of live parasites as described in the Materials and Methods.

from the blood of infected animals. This is routinely carried out in our laboratory by DEAE-cellulose chromatography, as described in the literature (Souza, 1983), because erythrocytes bind strongly to the positively-charged resin. However, since this does not occur with glass wool columns (unpublished data), they can not be used for the purification of bloodstream trypomastigotes.

The reasons for the easier elution of trypomastigotes from the different columns, as compared to epimastigotes are not clear. The present observation, that dead trypomastigotes are retained on glass wool columns to a much greater extent, could be explained by alterations in their surface characteristics increasing their affinity for glass. Alternatively, the formation of clusters of these parasites could interfere with their elution from the columns. The possibility that the 45-kDa glass-adherent surface protein, demonstrated in epimastigote amphiphilic preparations, could be present in a cryptic form in live trypomastigotes (and would therefore not be available for surface radioiodination) but exposed in dead trypomastigotes, (accounting for their binding to glass), should be taken into consideration. Whether this molecule would play a role in the binding of epimastigotes to other surfaces such as DEAE-cellulose, cotton wool, BIO-GEL P2 and tissue paper is not yet known. Furthermore, it has been recently reported that a group of proteins with M_F of 45–50 kDa is important in the adhesion process of epimastigotes during metacyclogenesis (Bonaldo

et al., 1988). The present findings that epimastigotes bind to glass wool in PBS-diluted LIT cultures, without being subjected to any additional manipulation, and that centrifugation of parasites in neat LIT does not increase their affinity for glass, argue against the possibility that putative alterations to the parasite surface due to the centrifugation process would be involved in the binding to glass.

Kanbara and Nakabayashi (1983) have shown that more than 90% of cell culture-derived *T. cruzi* trypomastigotes do not bind to the cation-exchange resin CM-cellulose in the presence of culture medium. This is very similar to the findings reported here concerning axenic culture-derived trypomastigotes and glass wool. Those authors also reported that *T. cruzi* amastigotes bind to CM-cellulose. A similar binding of amastigotes to glass wool was observed by us (unpublished data).

The contribution of ionic interactions to the binding of epimastigotes to DEAE-cellulose seems to be relatively small under the conditions used, since almost 90% of the epimastigotes remained bound to DEAE-cellulose in the presence of 10% BSA (Table 2). Since epimastigotes bind to non-charged BIO−GEL P2 (Hjerten and Mosbach, 1962), it is possible that their binding to DEAE-cellulose is not dependent on electric charge.

In conclusion, the separation of trypomastigotes from epimastigotes by the different columns described here could be due to at least two phenomena which are not necessarily exclusive: (1) binding of epimastigotes, or perhaps dead trypomastigotes, to the different materials through adhesive surface molecules and (2) ability of live trypomastigotes to escape from physical trapping in the columns more readily due to their higher mobility compared to epimastigotes and, of course, to dead trypomastigotes.

Acknowledgements

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