A.M. Fernández-Presas · J. Tay Zavala  
I. Becker Fauser · M.T. Merchant  
L. Robert Guerrero · K. Willms

Ultrastructural damage of *Trypanosoma cruzi* epimastigotes exposed to decomplemented immune sera

Received: 8 June 2000 / Accepted: 22 February 2001 / Published online: 12 May 2001  
© Springer-Verlag 2001

**Abstract** The susceptibility of *Trypanosoma cruzi* epimastigotes to lysis by normal or immune sera in a complement-dependent reaction has been reported, but the effects induced directly by immune serum depleted of complement remain unstudied. The aim of this work was to study the ultrastructural alterations induced in *T. cruzi* epimastigotes by immune mouse or rabbit sera with or without complement. A local isolate of *T. cruzi* (Queretaro) was used in all experiments. Immune sera were raised in both mouse and rabbit by immunization with *T. cruzi* epimastigote antigens. Light microscopy showed intense agglutination of epimastigotes when incubated with decomplemented mouse or rabbit immune sera. A distinctive ultrastructural feature of this agglutination pattern was the fusion of plasma membranes and a pattern of intercrossing between subpellicular microtubules. Agglutination was associated with fragmentation of nuclear membranes and swelling of cytoplasm, Golgi cisternae, endoplasmic reticulum, mitochondria and kintoplast membranes. Agglutinated parasites also incorporated trypan blue stain. Results of [*H*]-thymidine incorporation confirmed that epimastigotes exposed to specific antibodies in the absence of complement were incapable of proliferating. Ultrastructural changes observed in epimastigote micrographs incubated with decomplemented immune mouse sera were statistically significant (*P*< 0.001) when compared with results obtained from images after incubation with decomplemented normal mouse sera.

**Introduction**

Infective *Trypanosoma cruzi* trypomastigotes are resistant to lysis by normal sera from several mammalian species, whereas *T. cruzi* epimastigotes can be lysed by these sera in a complement-mediated reaction (Muniz and Boriello 1945; Rubio 1956; Nogueira et al. 1975). However, it has been reported that lysis of blood trypomastigotes can be observed in vitro when incubated with sera from Chagasic patients or from animals chronically infected with *T. cruzi* (Kretti and Brener 1982; Yoshida and Araguth 1987). Additionally, both sera from mice immunized with killed trypomastigotes (Yoshida 1986) and stage-specific monoclonal antibodies have been found to lyse metacyclic trypomastigotes in a complement-dependent reaction (Teixeira and Yoshida 1986). The incubation of *T. cruzi* epimastigotes with normal rabbit serum produces a fuzzy deposit on the parasite surface with loss of subpellicular microtubules; and negative staining reveals circular lesions, the result of damage by antibody-and-complement-mediated lysis (Kototani et al. 1979).

Excepting the destruction of *T. cruzi* trypomastigotes by avian serum, which is an antibody-independent process, lysis has been reported to be the result of complement activation by specific antibodies (Kierszenbaum et al. 1981).

Although the effect of antibodies and complement on epimastigotes has been widely studied, little is known about the role of specific antibodies alone on the transition forms or “epimastigote-like” stages in the mammalian host. The aim of this work was to study whether specific anti-*T. cruzi* antibodies obtained in mice and rabbits were able to induce ultrastructural alterations in *T. cruzi* epimastigotes in the absence of complement *in vitro*. 

---

A.M. Fernández-Presas (✉) · J.T. Zavala · M.T. Merchant  
L.R. Guerrero · K. Willms  
Departamento de Microbiología and Parasitología,  
Edificio A, 10 Piso, Facultad de Medicina,  
Universidad Nacional Autónoma de México,  
Cuidad Universitaria, 04510 México D.F., México  
E-mail: presas@servidor.unam.mx  
Fax: +52-55-3232382

I. Becker Fauser  
Departamento de Medicina Experimental,  
Facultad de Medicina, Universidad Nacional Autónoma de México,  
Cuidad Universitaria, 04510 México D.F., México
Materials and methods

Experimental animals

Female mice of the CD-1 strain, 4 weeks of age and weighing 20±2 g, and New Zealand white albino male rabbits, 16 weeks old and weighing 3±0.5 kg were used to obtain immune sera. They were maintained under a photoperiod of 12 h light and 12 h darkness, at a temperature of 21±2 °C and humidity of 55±10%, and were fed Purina laboratory chow.

Parasites

Trypanosoma cruzi were originally isolated from feces of a Triatoma barberi specimen from the State of Queretaro, Mexico, and were maintained in our laboratory for 10 years. Epimastigotes were grown at 28 °C in RPMI 1640 medium (Gibco, Grand Island, N.Y.) containing 10 U penicillin/ml, 25 μg streptomycin/ml (Gibco-BRL, Gaithersburg, Md.), 10 ml Hepes buffer (25 mM; Gibco, Grand Island, N.Y.), 200 ml t-glutamine (Sigma, St.Louis, Mo.) and 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, N.Y.).

Parasite antigens

Parasites were harvested at 5-8 days of culture (log growth phase), according to Powell and Kuhn (1980). Epimastigotes were washed three times by centrifugation at 750 g for 20 min in sterile phosphate-buffered saline (PBS) at 4 °C. Parasites were homogenized by seven cycles of freezing (at −82 °C), thawing and sonicating (10 min each cycle; model ME 21, Ladd).

Protein concentration of the homogenate was determined by the method of Lowry et al. (1951).

Hyperimmune sera

Antisera were obtained by immunization of CD-1 mice for 6 weeks at 8-day intervals. Antigen at a concentration of 100 μg/50 μl PBS was injected intraperitoneally for 6 weeks with Freund’s complete adjuvant in the first week, and with Freund’s incomplete adjuvant in the second week. Anesthetized mice were bled from the orbital sinuses 7 weeks after the first immunization.

To prepare immune rabbit sera, animals were inoculated subcutaneously at 18-day intervals for 8 weeks, using ten sites under the dorsal skin. Antigen at a concentration of 300 μg/500 μl PBS was injected with Freund’s complete adjuvant in the first inoculation and with Freund’s incomplete adjuvant in the second immunization. Sera were collected 9 weeks after the first immunization by bleeding from the marginal ear vein.

Antibody titers were estimated by enzyme-linked immunosorbent assay, according to Butler et al. (1978). Sera were pooled, aliquoted and stored at −80 °C until use. A second lot was heat-inactivated at 56 °C for 30 min. Sera obtained before immunization were used as controls.

Incubation of T. cruzi epimastigotes with immune and control sera

For light microscopy, epimastigotes obtained in the log growth phase were washed three times in RPMI 1640 by centrifugation at 750 g for 20 min. Suspensions of 5x10⁷ parasites/50 μl were incubated for 5, 30 and 60 min at 37 °C with 100 μl of mouse or rabbit serum, diluted 1:100 with or without complement. After incubation, aliquots of epimastigotes were deposited on glass slides, air-dried, stained with Giemsa and observed in a Nikon Optiphot light microscope. All incubations were performed in triplicate.

For electron microscopy, a suspension of 5x10⁷ parasites/50 μl was incubated for 5, 30 or 60 min at 37 °C with 100 μl of mouse or rabbit serum, diluted 1:100 with or without complement. After incubation, parasites were washed three times in fresh medium at 4 °C, rinsed in 0.15 M cacodylate buffer and fixed in Karnovsky’s solution for 1 h at room temperature (Karnovsky 1965). They were then transferred to 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated and embedded in PolyBed 812/DMP30 (Polysciences, Warrington, Pa.). Ultrathin sections were obtained with a diamond knife on a Reichert Ultracut S microtome, collected on copper grids and stained, first with 5% uranyl acetate in water and then with 0.25% lead citrate in 0.1 M NaOH (Reynolds 1963). Sections were photographed, using a JEOL JEM-1200 EXII transmission electron microscope.

Analysis of cell death

Two distinct criteria were used in order to explore epimastigote death. Epimastigotes exposed to mouse or rabbit normal or immune sera with or without complement were counted at different times points in a hemocytometer. Spheroid epimastigotes that had lost motility and parasites stained with (0.4%) Trypan blue dye (Sigma, St. Louis, Mo.) were considered dead.

Analysis of swelling

The analysis of ultrastructural swelling on epimastigote nuclei, plasma, kinetoplast and mitochondrial membranes was made by measuring the distance between membrane bilayers of ≤ 200 parasites under experimental and control conditions, using micrographs at a magnification of 60,000× with a × magnifier (Bausch & Lomb).

Three measurements were made of each membrane. To determine swelling of nuclei, the diameter was measured on light micrographs at magnifications of 400×.

Statistical analysis

Experimental and control conditions were statistically compared for significance using analysis of variance (ANOVA), followed by the Student–Newman–Keuls multiple comparisons test to allow comparisons between epimastigotes incubated with normal or immune mouse serum or RPMI 1640. The predetermined level of significance was P < 0.05. Statistical analysis was performed with the Instat Statistical program (GraphPad, San Diego, Calif.).

Incubation of epimastigotes with decomplemented mouse sera with [3H]-thymidine

Under the same conditions used for the ultrastructural analysis, epimastigotes were incubated with decomplemented immune or normal mouse serum diluted 1:1, 1:5 or 1:10 and incubated with 1.0 μmol Ci [3H]-thymidine (74 GBq/mmol; ICN, Irvine, Calif.) for 0.5, 24 and 48 h. Parasites were harvested onto glass microfiber filters (Whatman 934-AH) and washed with distilled water, using a multiple automated sample harvester (Nunc, Denmark). [3H]Thymidine uptake was measured by liquid scintillation counting (LS 6000 TA, Beckman, Fullerton, USA). Data were expressed as mean counts/min ± SE for each condition.

Results

Light microscopy

Epimastigotes incubated with decomplemented immune mouse serum agglutinated and formed webs (Fig. 1a). Exposure to whole immune mouse serum agglutinated the epimastigotes as early as 5 min, into compact clumps which increased in size over the next 30–60 min.