

Adhesion of *Trypanosoma evansi* to Red Blood Cells (RBCs): Implications in the Pathogenesis of Anaemia and Evasion of Immune System

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Abstract

Background: *Trypanosoma evansi* is the etiological agent of trypanosomiasis, a disease of domestic (horses, cattle and goats) and wild (capybara, vampires) animals characterized by anaemia, degeneration, necrosis and inflammatory processes. This disease is of great concern because it produces growth retardation, loss of body weight, low production of animal proteins and diminished fertility and traction power. Because anaemia is considered the most characteristic symptom, the aim of this study was to assess the effects that *T. evansi* adhesion to erythrocytes has on their morphology, surface oligosaccharides profiles and trypanosome surface antigens.

Methods: Blood and tissues samples from mice experimentally infected, were studied using scanning (SEM) and transmission (TEM) electron microscopes and lectin's histochemistry (sConA, sWGA, PNA, LFA, etc.). Furthermore immunoprecipitation of *T. evansi* radio iodinated surface antigens with specific anti-hosts and anti-trypanosome sera was performed and antigens analysed by electrophoresis and autoradiography.

Results: *T. evansi* adhesion to erythrocytes as well as changes in the morphology of them, were frequent findings. SEM studies showed that adhesion of bloodstream trypomastigotes of *T. evansi* to mature erythrocytes and reticulocytes occurred through its flagellum, undulating membrane or cellular body and were mediated by filopodia. Minute pores and filamentous material were sometimes observed on erythrocytes membrane at the point of adhesion to the trypanosome. Oligosaccharides changes of RBCs glycofocalix were characterized by a marked decrease in Man/Glc, terminal GalNAc and terminal Neu5Ac labelling accompanied by an increase in labelling of terminal GlcNAc. There were not labelling changes with the other lectins assayed. Preliminary immunoprecipitation studies of *T. evansi* radio iodinated surface antigens with anti-host sera, showed the presence of a of 45 kDa antigen from erythrocyte and host plasma on *T. evansi* plasma membranes. Furthermore, TEM studies demonstrated an increased abnormal erythrophagocytosis in adrenal gland, spleen and liver of experimentally infected mice.

Conclusion: Adhesion of *T. evansi* to erythrocytes together with morphological and biochemical changes of these cells must be responsible for abnormally incremented erythrophagocytosis. On the other hand, detection of antigens from host on *T. evansi* membranes, suggests the operability of the antigen masking as an alternate immune response evasion mechanism to antigenic variation described in African trypanosomes.

Keywords: *Trypanosoma evansi*; Cell adhesion; RBC; SEM; TEM; Erythrophagocytosis; Lectins; Surface Antigens; Antigen masking

Abbreviations: SEM: Scanning Electron Microscope; TEM: Transmission Electron Microscope; kDa: Kilodaltons; RBCs: Red Blood Cells; NMRI: Naval Medical Research Institute; IVIC: Instituto Venezolano de Investigaciones Científicas; Ip: Intraperitoneal; EDTA: Ethylene Diamine Tetra-Acetic Acid; DMSO: Dimethyl sulfoxide ;

PSG: Phosphate Saline Glucose Buffer; ABC: Avidin-Biotin Complex; DAB: Diaminobenzidine; sConA: Succinyl Concanavalina A; sWGA: Succinyl Wheat Germen Agglutinin; SBA: Soybean Agglutinin; UEA-I: *Ulex europaeus* Agglutinin I; SNA: *Sambucus nigra* Agglutinin; PNA: Peanut Agglutinin; LFA: *Limax flavus* Agglutinin; MAA or MAL-II: *Maackia Amurensis* Agglutinin or *Maackia Amurensis* Lectin II; D-Metil-man: D-Metil-mannose; D-Man: D-Mannose; D-Glc: D-

Glucose; GlcNAc: N-Acetyl Glucosamine; α - and β -GalNAc: α - and β -N-Acetyl Galactosamine; D-Gal: D-Galactose; D-Fuc: D-Fructose; Neu5Ac: N-Acetylneuraminic Acid; GalNAc: N-Acetyl Galactosamine; RaE: Rabbit Anti-Mouse Erythrocyte Membrane Serum; RaP: Rabbit Anti-Mouse Plasma Serum; RaT: Rabbit Anti-*T. evansi* serum; MaT: Mouse Anti-*T. evansi* serum; NR: Normal Rabbit Serum; NM: Normal Mouse Serum; DEAE-Cellulose: Diethyl-Amino-Ethyl Cellulose; KRT: Krebs Ringer Tris Buffer; PMSF: Phenyl Methyl Sulphonyl Fluoride; SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis; CPM: Counts Per Minute; ^{125}I : ^{125}I Iodine Isotope; PCV: Packed Cell Volume; LD: Lectin Domain; TconTS: *Trypanosoma congolense* Catalytic Domain of Transialidase; LacNAc: N-Acetyl Lactosamine; gp68: 68 kDa Glycoprotein; gp29: 29 kDa Glycoprotein; VSG: Variant Surface Glycoprotein.

Introduction

Of all the diseases caused by the different African trypanosomes species, infections produced by Venezuelan isolates of *T. evansi* and *T. vivax* have been the least studied. In Venezuela information concerning ultrastructural changes caused by these parasites in their host, as well as experimental animals is scarce. In this regard, Quinones-Mateu et al. [1] studied muscle changes in horses naturally infected with *T. evansi* in Hato El Frío (Apure State, Venezuela), and typical changes of autoimmune diseases, such as necrosis of skeletal muscle fibers and capillaries with mononuclear infiltration were found. Moreover, as a part of ultrastructural studies on the experimental infections of mice we described the development of intracellular forms of *T. evansi* [2,3] and varying degrees of ultrastructural abnormalities in cells of adrenal cortex [2], muscle fibers [4], hepatocytes [3], blood capillaries and endothelial cells [2,3,5].

Changes that occur in blood cells during infection by trypanosomes have been also studied. Anaemia is the most important finding in many trypanosomosis of veterinary importance [6] because it is a good indicator of infection severity. There is haemolytic in nature, occurring intravascularly in early stages of acute phase, and also extravascularly in sub-acute and chronic phase of disease [6].

Causes of anaemia in animal trypanosomosis are not entirely known, being haemolysis, hemodilution, bleeding and bone marrow dyserythropoiesis [7] the major events to which it relates. Haemolysis can be attributed to biologically active molecules produced by living or dead trypanosomes [8,9] as well as to mechanical damage that RBCs may experience due to the motility and adhesion of the flagellates to their surface [10].

Anaemia and systemic alterations caused by trypanosomes during infection are consequences of metabolic spoliation, mechanical cell injuries caused by trypanosomes motility and invasion of host's tissues [5], and secretion of biologically active macromolecules capable to produce biochemical alterations of cell surface. However effects of host's immune response against trypanosomes and the evasion mechanisms expressed by these flagellates in order to elude immune system act synergistically allowing colonization of host's body.

Evasion of host's immune system by African trypanosomes is achieved thanks to a well-studied and described genetic mechanism known as Antigenic Variation, however as will be discussed in this paper, the operability of other mechanisms described for haemoparasites such as antigen masking, extensively described for *Schistosoma mansoni* and other trypanosome species and protozoa,

could constitute an alternate mechanism of immune system evasion to antigenic variation.

This work is a contribution to the knowledge and understanding of pathogenic mechanisms that affects RBCs during murine experimental infections. The aim of this study was to assess the effects that *T. evansi* adhesion to erythrocytes has on their morphology, surface oligosaccharides profiles and trypanosome surface antigens. In order to reveal morphological, ultrastructural and histochemical changes that occur in RBCs during infection, different electron microscopy, histochemical, biochemical and serological techniques were used. Results obtained allowed to propose adhesion of *T. evansi* to RBCs as a pathogenic mechanism responsible for morphological alterations which in turn render them more prone to erythrophagocytosis. In addition results obtained from study of *T. evansi* surface antigens during infection suggest occurrence of an alternate mechanism of immune response evasion different to antigenic variation.

Materials and Methods

Experimental infections with *T. evansi*

A total of 15 heterozygous albino mice (20 g body weight) of the NMRI strain were used. Animals were purchased at the Venezuelan Institute for Scientific Research (IVIC) and kept in an experimental animal facility in cages with shell rice as a bed. They were fed ad libitum with Ratarina® (Protinal) and filtered ozonized water.

The heterogeneous population of *T. evansi* used in this work was isolated in 1991 from a horse (*Equus caballus*) infected at Hato El Frío (7°56' North latitude and 68°57' West longitude) in the Apure State of Venezuela. The infected horse showed signs of cachexia, prostration and paresis of the hindquarters knew as "Derrengadera" (equine trypanosomosis) in Venezuela.

The isolation was performed by intraperitoneal (ip) inoculation of horse blood aliquots in two albino mice of NMRI strain. When trypanosomes were patent in tail vein blood, a subpassage in Sprague-Dawley rats was done by inoculation. Murine parasitaemia was evaluated daily from tail vein blood by microhaematocrit method [11] and when values reached 10^7 - 10^8 trypanosomes/mL, the whole animal blood was obtained by heart puncture under anaesthesia, using 15% mg/mL EDTA (ethylene diamine tetra-acetic acid) as an anticoagulant. Part of blood obtained was cryopreserved with dimethyl sulfoxide (DMSO) in phosphate saline glucose (PSG) buffer pH 8 (20 mM phosphate, 150 mM NaCl, 1% glucose and 15% DMSO) at -196°C (liquid nitrogen).

Trypanosomes present in blood of infected horse and inoculated mice and rats, were characterized as monomorphic trypomastigote forms, and were classified as *T. evansi*, according to clinical, morphological (*T. brucei*-like) and biological (susceptibility for experimentation animals) criteria. Furthermore trypanosomes were classified as *T. evansi* according to its ultrastructure feature (low number of coated vesicles in the flagellar pocket in relation to *T. equiperdum* a co-endemic specie of trypanosome) [12].

After thawing a stabilate vial in a water bath at 40°C, 0.50 ml of cryopreserved blood was inoculated into Sprague-Dawley rats in order to expand parasitic populations. After rats reached parasitemias of 10^8 trypanosomes/ml an inoculum was prepared by diluting aliquots of infected blood in PSG [13] in order to inoculate mice with 10^4 trypanosomes (500 trypanosomes/g body weight). Parasitemias of

mice were evaluated daily from tail vein blood by Brenner method [14]. The deep circulation blood to be used in SEM studies was obtained by cardiac puncture using EDTA as anticoagulant. Samples of liver, spleen and adrenal glands to be studied by TEM were obtained from anesthetized animals. All the samples used were obtained at terminal stage of infection (parasitemia= 10^9 trypanosomes/mL).

Electron microscopy

For scanning electron microscopy (SEM) studies, when parasitemia of mice reached values around 10^8 - 10^9 trypanosomes/mL, aliquots of blood were prefixed in Karnovsky's solution (2.5% glutaraldehyde and 4% p-formaldehyde in Millonig's phosphate buffer 0.10 M pH 7.4), leaving the blood dripping gently on the fixing solution. The biological material was allowed to settle and after 2 hours of fixation at 4°C, excess of glutaraldehyde was removed by pipetting and washed 3 times with Millonig's buffer. The entire process was carried out by decantation to avoid the formation of cell aggregates. All next steps were performed according to Boada-Sucre et al. [15] methodology. Briefly 100 µL of prefixed blood were spotted onto coverslips pre-treated with poly-L-lysine 0.1% v/v in order to promote adhesion of blood cells to glass. Excess of glutaraldehyde was removed by washing with Millonig's phosphate buffer and post-fixation was performed with 1% w/v osmium tetroxide (OsO_4) for 1 hour at 4°C. Osmium excess was removed by washing with deionized water and samples were dehydrated by sequential incubation of glass coverslips in a battery of increasing ethanol concentration (50%, 70%, 80%, 90%, 95% and 100% at 4°C). Biological material adsorbed to coverslips was dried by evaporation on a critical point dryer (Hitachi HCP-2) using carbon dioxide (CO_2) as liquid transition fluid. The dried samples (coverslips) were mounted on a metallic sample holder with double-sided tape and silver paste and then were covered with platinum in a thermionic coater (Electron Microscopy Science EMS-350). Observations and photographic records were performed on a field emission scanning electron microscope (FE Hitachi S-4500) with an accelerating voltage of 5 kV.

After taking samples of adrenal glands, liver and spleen, pieces of these organs were processed according to technique described in [2] and ultrafine sections of them were obtained for transmission electron microscopy (TEM) study. In this regard, sample blocks of tissue-samples were subjected to ultramicrotomy in order to obtain sections of 60 to 90 nm thick, which were collected on copper grids of 300 mesh and contrasted with an aqueous solution of 1% w/v uranyl acetate for 30 minutes and lead citrate for 10 minutes according to the methodology described by Reynolds [16]. Observations and photographic records were performed on a field emission scanning electron microscope (Hitachi H-7100) with an accelerating voltage of 75 kV.

For freeze-fracture studies performed by TEM, blood aliquots from mice infected with *T. evansi* were prefixed in Karnovsky's solution, washed with Millonig's buffer and cryoprotected for 30 minutes by incubation in 10 mM phosphate buffer pH 7.2 containing 25% v/v glycerol. Cell suspensions obtained were placed in a sample holder, quickly frozen in liquid nitrogen, fractured, freeze-etched and covered with platinum-carbon, to finally obtain replicas after removal all organic material with bleach (3% v/v sodium hypochlorite) [17]. Replicas obtained by freeze-fracture were observed and photographic recorded in transmission electron microscopy (Hitachi H-500) with an accelerating voltage of 75 kV.

Lectin's Histochemistry

Oligosaccharide composition of RBC surface from healthy and *T. evansi* experimentally infected mice was studied by lectin's histochemistry technique described by Gonzalez-Elorriaga and Canepa [18]. Briefly, samples of adrenal glands, liver and spleen of infected animals and control group were fixed in 10% buffered formalin (pH 7.2) for 72 hours. Subsequently, fixed tissues were prepared according to conventional histological techniques. Histochemical reactions were performed on slides containing deparaffinized and hydrated 7 µm thick tissues sections. A total of seven biotinylated lectins with specificities for different configurations of specific carbohydrate residues in tissue (Table 1) were used at a final concentration of 10 µg/mL in 5 mM Tris-HCl pH 7.8 containing 150 mM NaCl, 0.10 mM $CaCl_2$, 0.10 mM $MgCl_2$, 0.7% w/v Carrageenan and 5% v/v Triton X-100. Lectin-Oligosaccharides were revealed by Avidin-Biotin Complex (ABC) method using diaminobenzidine (DAB)-hydrogen peroxide as chromogenic substrate. Results were observed under an optical microscope (Nikon OptiPlus) and scored semi quantitatively using a scale from 0 to 5 plus (+).

Source	Acronym	Inhibitors Saccharide Residues
<i>Canavalia ensiformis</i>	sConA	D-Metil-man >D-Man >D-Glc
<i>Triticum vulgare</i>	sWGA	GlcNAcβ(1→4)GlcNAcβ(1→4)GlcNAc
<i>Glycine max</i>	SBA	α- and β-GalNAc terminal>>D-Gal
<i>Ulex europaeus I</i>	UEA-I	Fuca(1→2)Galβ(1→4)GlcNAc>D-Fuc
<i>Sambucus nigra</i>	SNA	Neu5Ac-α(2→6)Gal and Neu5Ac-α(2→6)GalNAc
<i>Arachis hypogaea</i>	PNA	D-Galβ(1→3)GalNAc
<i>Limax flavus</i>	LFA	Neu5Ac and Neu5-Glc terminal
<i>Maackia amurensis</i>	MAA or MAL-II	Neu5Ac-α(2→3)D-Galβ

Table 1: Set of lectins used for determination of changes in RBC surface oligosaccharides composition.

Specific antisera

Specific antisera to be used in immunoprecipitation assays were prepared according to Simoes [19]. Specificity of these antisera: rabbit anti-mouse erythrocyte membrane (RaE), anti-mouse plasma (RaP) and anti- *T. evansi* (RaT), mouse anti- *T. evansi* (MaT), normal rabbit sera (NR) and normal mouse (NM) were done by Outcherlony technique (agar double immunodiffusion).

Purification of *T. evansi* Bloodstream Trypomastigotes and Preparation of RBC Ghosts

In order to perform surface radio iodination of *T. evansi*, blood trypomastigotes from Sprague- Dawley rats experimentally infected were obtained by anion exchange chromatography on columns of diethyl-amino-ethyl cellulose (DEAE-cellulose) according to technique described by Lanham and Godfrey [13]. RBC ghosts free of cytoplasm and haemoglobin were obtained from heparinized blood of normal mouse as it has been described by Newbold et al. [20].

Preparation of Plasma Membrane Fractions from *T. evansi* and Studies of Surface Antigens Composition

Changes in composition of *T. evansi* surface antigens during experimental infections of mice were determined by preparation of a radio iodinated cell membrane fraction, and immunoprecipitation of *T. evansi* surface antigens with specific antisera and Protein A-agarose. Radio-iodination of surface proteins with ^{125}I was performed with 2×10^8 intact blood trypomastigotes obtained by DEAE-cellulose chromatography, using Iodogen method [21]. Plasma membrane fractions from bloodstream trypomastigotes (2×10^8) were obtained after 1 hour lysis at 4°C in Krebs Ringer Tris (KRT) pH 7.4 containing 1% Triton X-100, 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF) and 1 mM EDTA (ethylene diamine tetraacetic acid) followed by centrifugation for 30 minutes at 38,000 x g. Immunoprecipitation with specific antisera was performed overnight at 4°C by mixing 50 µl of radio iodinated fractions with 40 µl of specific antisera in 10 µl of 50 mM Tris-HCl pH 8.2 containing 150 mM NaCl, 10 mM EDTA and 1 mM PMSF. Overnight incubation was followed by an additional incubation of 1 hour with gentle mixing followed by 3 minutes boiling of fractions mixed with 50 µl of 2X sample buffer for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [22]. Surface antigens were then separated in 12.5% SDS-PAGE under dissociating conditions [22], stained and dried in order to perform autoradiography.

Results

SEM of blood from *T. evansi* experimentally infected mice (Figure 1) revealed occurrence of abnormalities of RBCs characterized by presence of holes (Panel A), rough surfaces (Panel B and D) and donut-shaped RBCs (Panel C).

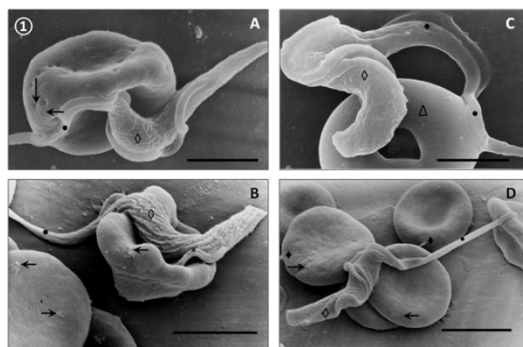


Figure 1: Scanning electron micrographs of *T. evansi* adhesion to RBCs from experimentally infected mice. Panel A: Occurrence of tiny holes (→) in the surface of a deformed RBC adhered to a bloodstream trypomastigote (◇) through flagellum (★); Bar: 3 µm. Panel B: Adhesion of a bloodstream trypomastigote to a mice RBC through cell body (◇), undulating membrane (★) and flagellum (★), showing RBCs warty surfaces (→); Bar: 3 µm. Panel C: Adhesion of a bloodstream trypomastigote (◇) to a donut-shaped RBC (△) through flagellum (★); Bar: 3 µm. Panel D: Electron micrograph showing three RBCs adhered to *T. evansi* through flagellum (★) and cell body (◇). Notice the presence of warty surfaces (→) and the emission of RBC filopodia (★); Bar: 4 µm.

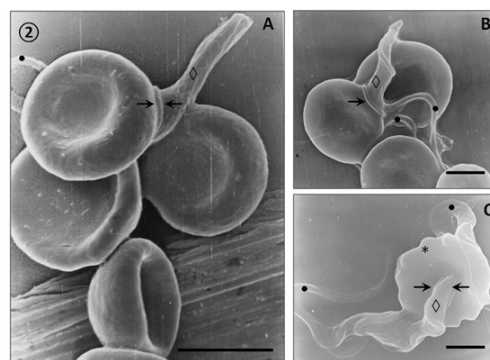


Figure 2: Clusters of RBCs adhered to *T. evansi* during experimental infection of mice. Panel A and B: Adhesion (→) of *T. evansi* (◇) to more than one RBC determining formation of large aggregates or trypanosomes-RBC microthrombi; (★) flagellum; Panel A Bar: 5 µm; Panel B Bar: 1.5 µm Panel C: Bloodstream trypomastigote of *T. evansi* (◇) passing through (→) a RBC body (★); (★) flagellum; Bar: 2 µm.

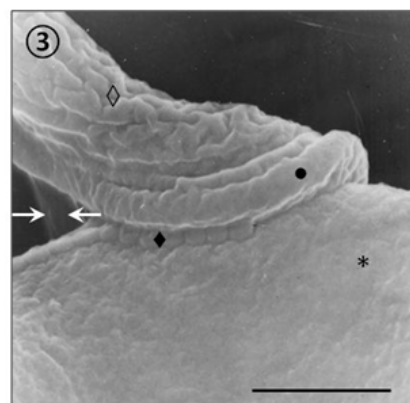


Figure 3: Scanning electron micrographs of cell surface interaction between a trypomastigote of *T. evansi* and a RBC. High magnification scanning micrograph showing the emission of trypanosomal-filamentous material (→←) in the vicinity of a trypanosome (◇)-RBC; (*) adhesion through a zip-type connection sealing structure (★); flagellum (★); Bar: 720 nm.

Adhesion of *T. evansi* to RBCs was through cell body and undulating membrane (Panels A, B and C) as well as flagellum (Panel D). In all preparations studied, adhesion of trypanosomes to RBCs was observed to one (Panel A and B) or more RBC (Figure 2) determining formation of large aggregates or trypanosomes-RBC microthrombi (Panels A and B). A rare finding was the presence of trypanosomes crossing RBC body (Figure 2, Panel C). Adhesion appears to be mediated by secretion of a filamentous material (Figure 3) and the emission of filopodia from trypanosomes and erythrocytes (Figure 4; Panels A and B). Figure 3 shows that binding of *T. evansi* to the RBC surface was intimate, determining the formation of zip-type connection sealing structure. Figure 4 shows that formation of this zip-

type connection (Figure 3) seems to be favoured by filopodia in the vicinity of the contact area, whose emission begins with formation of small bubbles on the RBC surface.

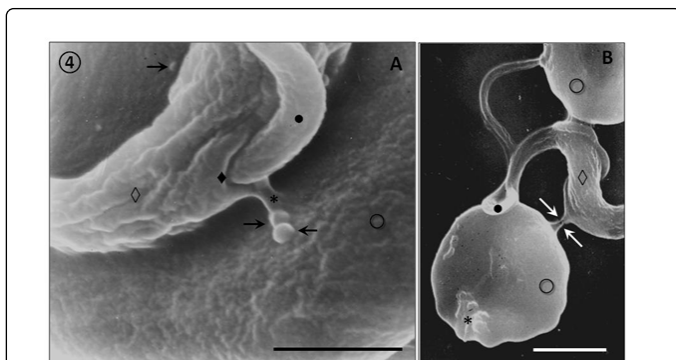


Figure 4: Emission of filopodia during *T. evansi*-RBC adhesion. Panel A: High magnification scanning micrograph showing the formation of RBC filopodia ($\rightarrow\leftarrow$) on its surface in the intimate area of *T. evansi* (\diamond) adhesion to RBC (\circ). Notice the apparent fusion of membrane (*) from *T. evansi* flagellar-pocket (\blacklozenge); (\bullet) flagellum; Bar: 720 nm. Panel B: Filopodia mediated adhesion of *T. evansi* (\diamond) to an abnormal RBC (\circ). Notice the binding of a filopodium emitted from RBC with another one from undulating membrane of *T. evansi* ($\rightarrow\leftarrow$); (*) Wartened surface; Flagellum (\bullet); Bar: 5 μ m.

Results from TEM revealed close relationships between trypomastigotes and deep circulating RBCs of adrenal glands (Figure 5, Panel A) and blood (Panels B, C and D), as well as of spleen and liver (not showed). Panel A shows presence of an electron dense material in the RBC-trypanosome contact apparently coming from trypanosome surface coat. Freeze-fracture replicas from deep-circulating RBCs confirmed this superficial close interaction (Panel B and C). In some cases was a frequent finding the continuity between RBC and *T. evansi* plasma membranes and the presence of lysosome-like organelles in the proximity of trypanosome-RBC interaction (Panel C). Other frequent finding in ultrathin sections (Figure 6) was observation of abnormal and increased RBC phagocytosis by macrophages in adrenal gland (Panel A), K \ddot{u} ppfer cells lining hepatic sinusoids (Panel B), and spleen macrophages (not showed).

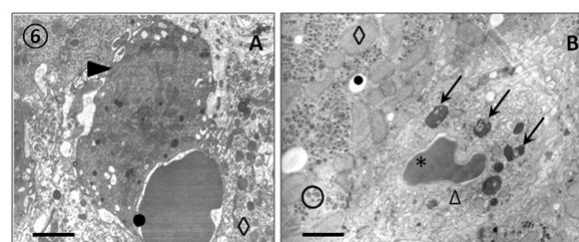


Figure 6: Thin sections of adrenal cortex and liver parenchyma of mice experimentally infected with *T. evansi*. Panel A: RBC phagocitized by a macrophage (aa) extravasated in adrenal cortex (\diamond). Notice the engulfed RBC inside a big vacuole (\bullet); Bar: 1.4 μ m. Panel B: Section of an hepatic sinusoid showing phagocytosis of a RBC (*) by a K \ddot{u} ppfer cell (Δ). Notice the presence of RBC in different stages of digestion inside secondary lysosomes (\rightarrow); (\bullet) Lipid droplets; (\diamond) Hepatocyte mitochondria; (\circ) Glycogen granules; Bar: 0.8 μ m.

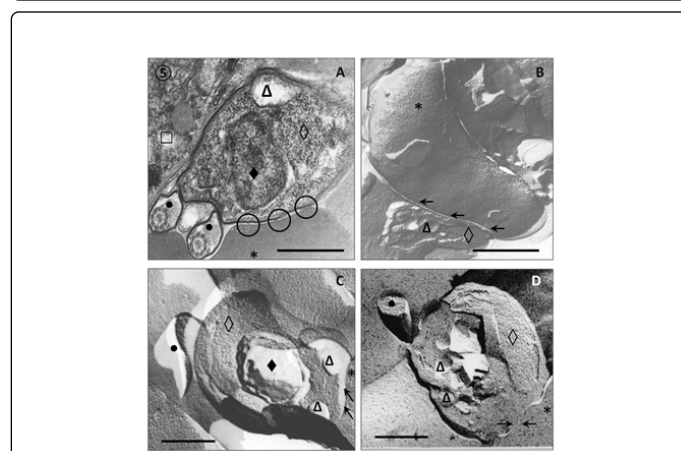


Figure 5: Transmission Electron Micrographs of *T. evansi* interactions with RBCs. Panel A: Interaction of a trypomastigote (\diamond) with RBCs (*) in a blood vessel of adrenal gland. Notice the presence of an electron dense material at trypanosome-RBC limits (\circ); (\bullet) Flagellum; (Δ) Mitochondrion, (\blacklozenge) Cell nucleus with nucleolus; (\square) Cortical cell; Bar: 0.70 μ m. Panels B, C and D: Freeze-fracture replicas of *T. evansi* adhesion to RBCs. Panel B and C: Intimate interaction (\leftarrow) between a trypomastigote (\diamond) and a RBC (*); (Δ) Lysosome-like profiles; (\bullet) Cell nucleus with nuclear pores; Panel B Bar: 1 μ m; Panel C Bar: 1.2 μ m. Panel D: Adhesion of a *T. evansi* trypomastigote (\diamond) with a RBC (*). Notice the continuity between trypanosome and RBC (*) membranes suggesting the occurrence of cell fusion ($\rightarrow\leftarrow$); (Δ) Lysosome-like profiles; Flagellum (\bullet); Bar: 1.3 μ m.

(Figure 7) shows results of histochemical staining of RBC's surface with a panel of seven specific lectins. Results scored allowed to verify occurrence of changes in composition of RBCs surface oligosaccharides during infection with *T. evansi*.

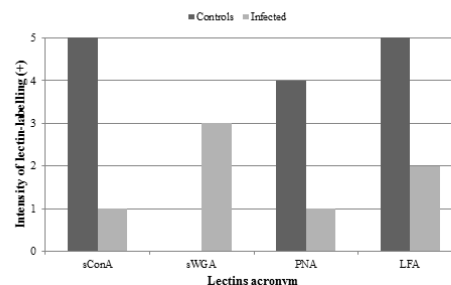


Figure 7: Changes in surface lectin-labelling of RBC's oligosaccharide during experimental infection of albino mice with *T. evansi*. (sConA: Succinyl Concanavalina A, sWGA: Succinyl Wheat Germen Agglutinin; PNA: Peanut Agglutinin; LFA: *Limax flavus* Agglutinin).

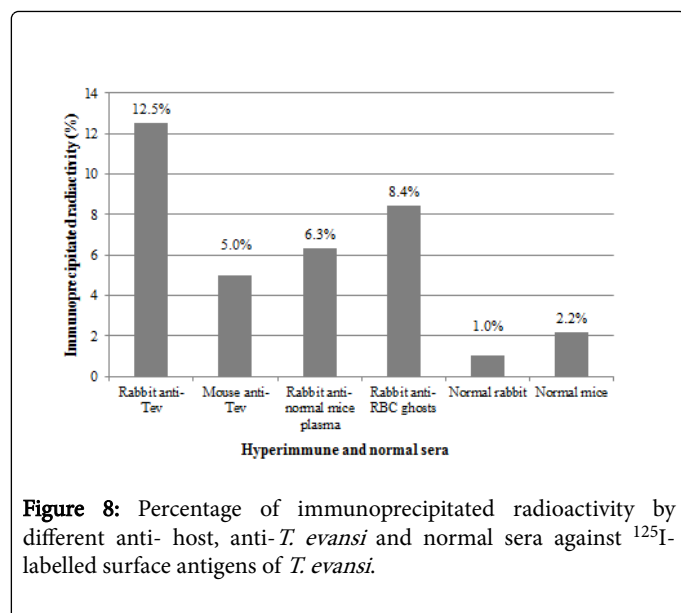


Figure 8: Percentage of immunoprecipitated radioactivity by different anti- host, anti-*T. evansi* and normal sera against ¹²⁵I-labelled surface antigens of *T. evansi*.

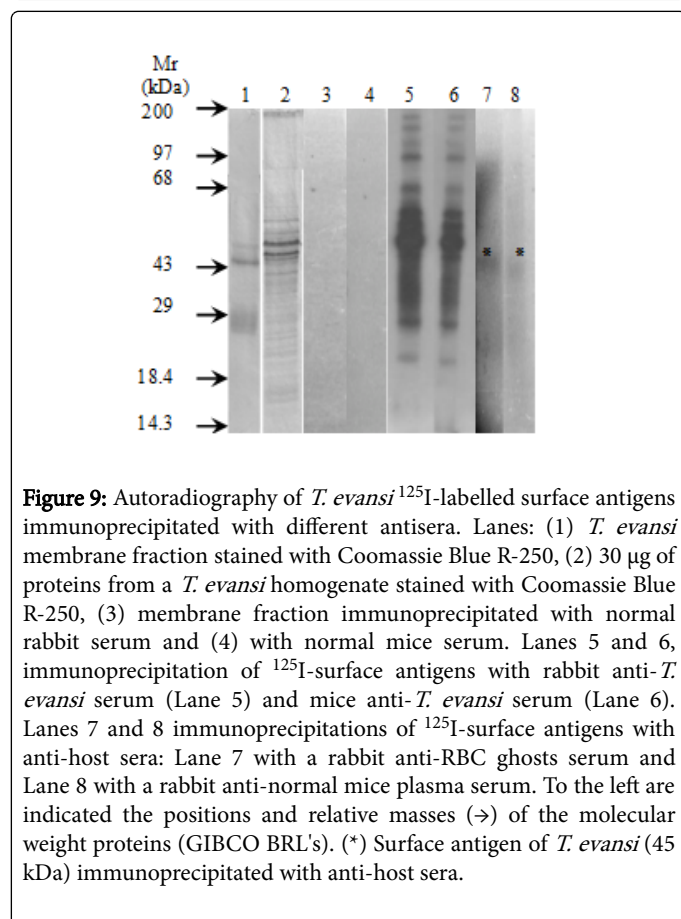


Figure 9: Autoradiography of *T. evansi* ¹²⁵I-labelled surface antigens immunoprecipitated with different antisera. Lanes: (1) *T. evansi* membrane fraction stained with Coomassie Blue R-250, (2) 30 µg of proteins from a *T. evansi* homogenate stained with Coomassie Blue R-250, (3) membrane fraction immunoprecipitated with normal rabbit serum and (4) with normal mice serum. Lanes 5 and 6, immunoprecipitation of ¹²⁵I-surface antigens with rabbit anti-*T. evansi* serum (Lane 5) and mice anti-*T. evansi* serum (Lane 6). Lanes 7 and 8 immunoprecipitations of ¹²⁵I-surface antigens with anti-host sera: Lane 7 with a rabbit anti-RBC ghosts serum and Lane 8 with a rabbit anti-normal mice plasma serum. To the left are indicated the positions and relative masses (→) of the molecular weight proteins (GIBCO BRL's). (*) Surface antigen of *T. evansi* (45 kDa) immunoprecipitated with anti-host sera.

Changes were characterized by a marked decrease in labelling of RBC's surface oligosaccharides containing Man/Glc by *Canavalia ensiformis* lectin (ConA), terminal GalNAc by peanut agglutinin (*Arachis hypogaea*) (PNA) and terminal Neu5Ac by *Limax flavus* agglutinin (LFA). These changes were accompanied by an increase in labelling of terminal GlcNAc residues with *Triticum vulgare* agglutinin (sWGA). There were not changes in the intensity of

labelling (+3) with the other three lectins assayed (SBA, UEA-I, SNA and MAA) when RBCs from infected mice were compared with those from control mice.

Specificity of antisera used in this work showed identity reactions with different fractions of *T. evansi* antigens by Outcherlony technique (results not shown). (Figure 8) shows percentages of radioactivity (cpm) immunoprecipitated from fractions of ¹²⁵I- *T. evansi* surface antigens using these specific antisera. As it can be seen rabbit anti- *T. evansi* antiserum precipitated 12.5% of radioactivity, followed by rabbit antiserum against to RBC ghosts (8.4%) and rabbit antiserum against normal mice plasma (6.3%). Mice anti- *T. evansi* serum precipitated 5% of radioactivity, while normal rabbit and mice sera precipitated non-specifically between 1 and 2.2% of radioactivity (Figure 9).

Discussion

Haematological abnormalities in infections caused by African trypanosomes have been extensively studied and the existing literature about pathogenic mechanisms involved was reviewed by several authors [23]. Among the most striking changes reported, decreased RBC count, packed cell volume (PCV) and morphological abnormalities of RBC seem to be best indicators that have been associated to anaemia that develops during the disease.

In the early 70's it was reported that morphological abnormalities of RBCs during trypanosomiasis are a consequence of changes that occur in small blood vessels which become abnormally thick and adopt tortuous shapes, affecting RBCs morphology and integrity [24]. Adhesion of *T. evansi* to mice RBCs and morphological changes of erythrocytes described in this paper such as perforations, warty surface, donuts-shape cells and emission of filopodia, are quite similar to those described in infections of deer mice (*Peromyscus maniculatus*) with *T. brucei* in absence of blood vessels damage [25].

Perforations on the surface of RBC's could be explained by the mechanical damage that bloodstream trypomastigotes cause to their membranes, especially when parasitemias are too high as 10⁸ - 10⁹ trypanosomes/ml. In this regard, mechanical damage "pinching out" caused by bloodstream forms of *T. evansi* has been demonstrated by cinematographic techniques with Normansky light interference, and may be a factor that contributes to characteristic haemolysis of African trypanosomiasis [10] and less frequently to occurrence of bloodstream trypomastigotes of *T. evansi* going through RBC as it has been described in this study. In addition activation of effector mechanisms of the immune system as a consequence of formation and adsorption of antigen-antibody complexes and complement on RBC's surface, could explain these injuries as well as the erythrolysis, their antigenic recognition and removal of RBCs by mononuclear phagocytic system [26].

Morphological changes of RBCs have been also described in lectins-treated erythrocytes. RBC membranes have internal protein skeletons that govern cells shape and morphology changes (discocyte-echinocyte) usually seen in conventional microscopy. Glycophorin (the RBCs transmembrane glycoprotein) presents all of its oligosaccharides outside cell and is intracellularly linked to the RBC cytoskeleton, enabling lectins binding to the external saccharides to gain profound control over internal cytoskeleton behavior expressed by governance of the visibly seen cell shape [27].

Decrease in lectin's labelling of RBCs from mice experimentally infected with *T. evansi*, such as sialic acids (desialation), not only

implies removal of this saccharide residues from erythrocyte glycocalyx but also the exposition of endosaccharides on RBC's glycoporphin enabling lectins to gain a tight control over RBCs morphology in simple stoichiometric ratios of bound lectin molecules/glycophorin receptor [27].

Lectin-like molecules have been described in African trypanosomes, in this regard, transialidase from *T. congolense* is an enzyme composed by two domains, a Lectin domain (LD) and a catalytic domain (TconTS) whose enzymatic activity over several (foreign and own) Man-containing oligosaccharides, such as mannobiose, mannotriose and higher mannosylated glycans, as well as Gal, GalNAc and LacNAc containing oligosaccharides is modulated by the LD domain [28]. Although transialidases have not been described in *T. evansi* and other trypanosomes of brucei-group, the presence of lectin-like molecules on its surface could also contribute together with other factors to morphological changes described for RBCs during experimental infection.

Adhesion of *T. evansi* to peripheral and deep circulation RBCs of adrenal glands, liver and spleen, seems to be a shared character with *T. brucei* [25] and other African trypanosomes. During adhesion of *T. evansi* to RBC's it was observed involvement of undulating membrane and flagellum as it has been described in flagellum-mediated adhesion of *T. congolense* to bovine aortic endothelium [29] and *T. vivax* to ovine RBCs [26]. This process was accompanied by emission of erythrocyte's and trypanosome's filopodia, cytoplasmic extensions that were described for the very first time in bloodstream trypomastigotes of *T. brucei* brucei, *T. brucei rhodesiense* [30] and *T. evansi* [31], and act as elements that promote adhesion of bloodstream trypomastigotes to RBCs [32].

Adhesion of *T. evansi* trypomastigotes to RBCs occurs with one or more erythrocytes, to give rise to formation of aggregates or microthrombi with an important role in trypanosomosis systemic pathology. In this regard, complications triggered by anaemia together with microthrombi of parasitic origin not only compromise the normal gas and metabolites exchange between blood and host tissues by way of the occlusion of the vascular lumen of capillary, but also contribute to the cell necrosis aggravated by endothelial alterations that occur in visceral organs during disease [2,3].

Another peculiar feature of *T. evansi* adhesion to mice RBCs were electron microscopic evidences of secretion of a filamentous material in the contact zones between trypomastigotes and RBCs. In this regard, electron microscopic evidences during adhesion of *T. congolense* to bovine aortic endothelial (BAE) cell monolayers [29] suggested that this filamentous material is constituted by proteins from trypanosome surface coat that are shed to cell culture. Furthermore, lectin-gold labelling assays demonstrated that contains Sialic acid residues involved in adhesion.

In *T. evansi* adhesion to RBCs this filamentous material could be constituted by two glycoproteins: a cysteine-proteinase of 29 kDa (gp29) with gelatinolytic activity which has been identified in secretion supernatants and homogenates of *T. brucei* [33] and Venezuelan isolates of *T. evansi* [34] and another one of 68 kDa (gp68) whose biochemical characterization on the basis of its molecular weight and recognition by lectins corresponds to the Variant Surface Glycoprotein (VSG) [34].

Although it is unclear how adhesion determines different kind of abnormalities described by SEM and TEM, we suggest that adhesion of *T. evansi* to RBCs via Sialic acid receptors causes injuries to RBCs

membrane [26] and should promote haemolysis and erythrophagocytosis in a direct association to parasitemia, since high trypanosomes densities in bloodstream at any given time increases the probability of contacts between parasites and RBCs [25,26]. This association is supported by the fact that increasing trend in parasitemia during the infection overlaps with the sharpest decline of the haematocrit during the experimental infections of cattle and goats with *T. evansi* [35], mice with *T. brucei* [23] and sheep with *T. vivax* [36].

Adhesion mechanisms through *T. evansi* can damage RBCs included also changes in the composition of surface oligosaccharides described by lectin's histochemistry. In this regard it has been found that during infections, live or dead trypanosomes produces a variety of biologically active molecules capable of producing modifications of host cells glycoalyx and cell damage [9]. Extracellularly-secreted cysteine proteinase (gp29) of *T. evansi* [34] shared with *T. brucei* and *T. equiperdum* [33,37] could act together with gp68 inducing not only the biochemical changes of RBC's surface but also cytoskeletal changes responsible for the morphological alterations described in this work. In this regard, it has been demonstrated that brucipain-like proteases induce changes in actin cytoskeleton of endothelial cells from central nervous system that allow *T. brucei gambiense* to enter into and trespass haemato-encephalic barrier in a process dependent on level of brucipain expressed by trypanosomes [38]. If to this process is added the fact that increased trypanosome motility during parasitemia peaks constitutes a mechanical stimulus that can trigger the release of intracellular Ca^{2+} followed by changes in actin cytoskeleton [38], operability of similar processes triggered by trypanosome swimming and secretion of an homologous cysteine proteinase (evansin), could explain morphology changes of RBCs such as donuts-shape cells, emission of filopodia from RBCs and the presence of trypanosomes crossing RBCs.

These processes could act synergistically with other biologically active molecules such as proteases and phospholipases from live or dead trypanosomes which could act favouring degradation of glycoproteins and other glycoconjugates from the RBC membranes, altering their oligosaccharides profiles and making them more prone to lysis and its removal by phagocytosis [9]. In this regard, this work provides evidences about changes in oligosaccharides composition of RBC surface, related to a decreasing of terminal D-Man/D-Glc, GalNAc and sialic acid, and an increase in labelling of terminal GlcNAc residues with *Triticum vulgare* Agglutinin (sWGA). These changes could be a consequence of glycosidases secreted by trypanosomes [39-42] such as sialidases [41] and β -galactosidases [42], enzymes which have been described and kinetically characterized in bloodstream trypomastigotes of *T. evansi*.

β -galactosidase catalyzes the hydrolysis of β -galactosides from lectins, glycoproteins and gangliosides. So far, among all the species of trypanosomes, β -galactosidase has only been described in *T. evansi* [42], *T. congolense* [43] and *T. cruzi* [44]. The enzyme has also been purified and characterized in *Trichomonas foetus* [45] where it is required for mucin breakdown during invasion process [46].

Its mechanism of action is in part due to action of sialidases whose levels are marked increased in plasma of bucks experimentally infected with African isolates of *T. evansi* [41]. This enzyme independently they come from living or dead trypanosomes, produces cleavage of surface sialic acids from erythrocytes, exposing β -galactosyl residues which in turn could be breakdown by β -galactosidase or been recognized by β -D-galactose specific lectins on the surface of adrenal cortex

macrophages and Kupffer cells from hepatic sinusoids, leading to the abnormal extravascular erythrophagocytosis observed in this work and responsible for the subsequent anemia developed [47,48].

Detection of a 45 kDa polypeptide from RBC and host plasma in plasma membrane fraction of *T. evansi* provides antigenic evidences about the occurrence of an unusual mechanism of immune evasion in Africa trypanosomes. The mechanisms that trypanosomes of brucei-group have developed to escape immune surveillance are currently under investigation in many laboratories, because their elucidation may provide new approaches to the prevention of the diseases that parasites cause [49]. One possible way of evasion supported by results herein presented is the uptake of host antigen by trypanosomes. This phenomenon of masking of *T. evansi* surface antigens by host proteins has been extensively studied and documented in schistosomiasis. In this regard, the acquisition of mouse antigens, human A, B, and H blood group antigens, glycolipids from erythrocytes, serum factors and mouse histocompatibility antigens have been demonstrated in *Schistosoma mansoni* and offer an effective mechanism for antigenic mimicry of host and subsequent escape from the immune response [50,51].

This mechanism would allow *T. evansi* to evade the immune system by a different mechanism than the antigenic variation previously described by Perrone et al. [52] for Venezuelan isolates as well as for *T. evansi* and other African trypanosomes [49,53]. Masking of host antigens, such as immunoglobulins has been described for bloodstream forms of many trypanosomes such as *T. congolense*, *T. brucei gambiense*, *T. brucei rhodesiense*, *T. cruzi*, *T. musculi* and *T. lewisi*. Other host proteins, such as albumin, have also been detected on surface of protozoa and some trypanosomes species. In this respect it has been suggested that surface immunoglobulins are responsible for the persistence of *T. lewisi* in the bloodstream of rats.

The acquisition of host antigens by *T. evansi* could be achieved by absorption from host plasma but also during *T. evansi*-RBC surface interactions and adhesion. In this regard, freeze-fracture replicas results showed areas in which trypanosome-RBC contacts appear as continuity regions between the plasma membrane of *T. evansi* and RBC. A similar feature of the adhesion phenomenon has also been described during cytoadherence of *Trichomonas vaginalis* to epithelial cells of the human vagina [54], *T. brucei* to RBCs [21] and *T. congolense* to endothelial [22] and RBCs [25]. These intimate contacts trypanosome-RBCs together with presence of filamentous material shed from the surface coat of trypanosomes to RBCs membrane and interaction of RBCs filopodia with those produced by *T. evansi* could account for the antigenic modifications of RBCs (not studied in this work), and those described for trypanosomes.

Conclusion

This work not only reports for the very first time, the adhesion of *T. evansi* to RBCs in a process mediated by emission of a filamentous material derived from surface coat and filopodia from trypanosomes and erythrocytes, but also fusion of plasma membranes between trypanosomes and RBC, changes in the oligosaccharide composition of RBC plasma membrane and antigenic modification of plasma membrane of *T. evansi*. While changes in antigenic composition of *T. evansi* plasma membrane could allow it to evade immune system by masking their own antigens with antigens taken from host, changes in RBC morphology and membrane oligosaccharides, surely contribute to changes in RBC antigenicity (not studied in this work) that determine

cell damage and lysis, as well as abnormal phagocytosis in adrenal glands, skeletal muscle, liver and spleen of experimentally infected mice. However, further research are needed to: (1) identify *T. evansi* antigens that could be responsible for the modification of RBC surface, (2) define the role that biologically active molecules secreted by *T. evansi* have on morphological and biochemical changes of host cells surface, (3) know the potential role of antigen masking on immune system evasion and (4) know precise nature of the adsorption of host antigens to plasma membrane of *T. evansi*.

Conflicts of Interests

The authors (M S Rossi S; A A Boada-Sucre; M T Simoes; Y. Boher; P Rodríguez; M Moreno; M Ledezma de Ruíz; M L Márquez; H J Finol, C Sanoja and G Payares) declare that there are no competing interests regarding the publication of this paper.

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