Acta Tropica 109 (2009) 219–225

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

# Antiproliferative effect of sera from chagasic patients on *Trypanosoma cruzi* epimastigotes. Involvement of xanthine oxidase

Susana M. Hernandez[a], Rodolfo A. Kolliker-Frers[b], Marcela S. Sanchez[c], Gabriela Razzitte[b], Rodny D. Britos[a], Maria E. Fuentes[a], Martha N. Schwarcz de Tarlovsky[a,*]

[a] *School of Medicine, CAECIS, Universidad Abierta Interamericana, Montes de Oca 745, 1270 Buenos Aires, Argentina*
[b] *Laboratory of Parasitology, Jose Maria Ramos Mejia Hospital, Urquiza 609, 1211 Buenos Aires, Argentina*
[c] *Ciclo Básico Común, Universidad de Buenos Aires, Paraguay 2155, 1121 Buenos Aires, Argentina*

ARTICLE INFO

*Article history:*
Received 20 August 2008
Received in revised form
10 November 2008
Accepted 14 November 2008
Available online 25 November 2008

*Keywords:*
*Trypanosoma cruzi*
Chagas disease
Xanthine oxidase
Reactive oxygen species
Hydrogen peroxide

ABSTRACT

Serum from asymptomatic or symptomatic (with cardiovascular manifestations) chagasic patients depleted of the complement system displayed an antiproliferative effect on *Trypanosoma cruzi* epimastigotes, RA strain, when added to the growth medium. This effect was also observed when patient's serum was depleted of specific antibodies.

The antiproliferative effect was both time and concentration dependent. It was confined to the non-dialyzable, high molecular weight, fraction of the serum. This effect was abrogated by allopurinol and catalase, and enhanced by N-ethylmaleimide. Xanthine oxidoreductase and xanthine oxidase activities were increased in the chagasic sera, while catalase activity remained unchanged. Parasites exposed to chagasic sera showed a decrease in Fe/superoxide dismutase activity as well as an increase in membrane lipoperoxidation. Our data provides evidence to support the idea that the antiproliferative activity observed in sera from chagasic patients may be due, at least partially, to a direct effect of hydrogen peroxide on the epimastigotes of *T. cruzi*. The increase of hydrogen peroxide to antiproliferative levels might result from an increase in xanthine oxidase activity in the serum of patients infected with the parasite.



## 1. Introduction

The trypanosomiases are a group of diseases affecting humans and livestock in Africa, Asia and South America. These diseases are caused by the infection of blood-dwelling protozoan parasites called Trypanosoma. *Trypanosoma brucei* subspecies *T. brucei gambiense* and *T. brucei rhodesiense* are the etiologic agents of sleeping sickness (African trypanosomiasis) while *T. cruzi* is the causative agent of Chagas disease (American Trypanosomiasis). Both parasites developed the genetic variability needed for survival in their host, by using different strategies. *T. brucei* changes periodically the expression of a group of variant surface glycoproteins (Cross, 1990; Vanhamme and Pays, 2004), whereas *T. cruzi* counts on the extreme heterogeneity of their population. The source of *T. cruzi* antigenic diversity may arise from metabolic changes in the mismatch repair pathway (Machado et al., 2006).

Nevertheless human blood, unlike the blood of other mammals, has an efficient trypanolytic activity against *Trypanosoma brucei brucei*. This is due to the presence of non-immune serum factors that lyse the invading trypanosome. One lytic factor of human serum against *T. brucei brucei* was characterized as a subset of high density lipoproteins (HDL) which invariably include haptoglobin related protein and apolipoprotein L1 (Raper et al., 1999; Drain et al., 2001; Pays et al., 2006). On the other hand, it has been observed that only Cape buffalo, among a wide variety of domestic animals, is resistant to infection by African trypanosomes. This resistance is due to accumulation of a trypanocydal concentration of hydrogen peroxide in the buffalo serum, during catabolism of xanthine by xanthine oxidase as a consequence of the infection (Muranjan et al., 1997; Black et al., 2001).

Infection, as well as various forms of tissue damage, induces inflammatory reactions as an important part of innate immunity. The inflammatory reaction results in the expression of a number of cytokines. Among these cytokines, gamma-interferon (IFN-γ), alpha-interferon (IFN-α), tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-3 (IL-3) stimulate xanthine oxidoreductase (XOR) expression (Berry and Hare, 2004).

XOR is a housekeeping enzyme with a role in purine catabolism, detoxification and the regulation of the cellular redox potential. The XOR enzyme is a homodimer composed of catalytically independent subunits with an approximate molecular mass of 150 kDa each. It exists in two inter-convertible enzymatic forms, as xanthine dehydrogenase (XDH) (E 1.1.1.204) the primary gene product of XOR and as xanthine oxidase (XO) (1.1.3.22), a product of

* Corresponding author. Tel.: +54 11 4301 5240; fax: +54 11 4301 5240x107.
*E-mail address:* marta.schwarcz@vaneduc.edu.ar (M.N. Schwarcz de Tarlovsky).

0001-706X/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.actatropica.2008.11.013

post-translational modifications of XDH. XDH favors the cofactor $NAD^+$ as its primary electron acceptor, while XO is unable to bind to $NAD^+$ and uses $O_2$ as its electron acceptor. Through both forms, but particularly through catalysis by the XO form, numerous reactive oxygen species (ROS) are synthesized. The ability of XOR to rapidly convert from XDH into XO under the effect of the same cytokines that stimulate XOR expression in response to tissue damage makes XOR an ideal component of fast innate immune response (Vorbach et al., 2003; Martin et al., 2004).

About 20 million people in America are infected with *T. cruzi* and 50,000 deaths per year are associated with the infection (World Health Organization, 2002). Our current study revealed that serum obtained from patients diagnosed with Chagas' disease displays an inhibitory effect on the growth of epimastigotes of the *T. cruzi* RA strain when added to the growth medium. This activity was not present in the serum of healthy individuals, nor was it due to antibodies against the parasite or to the complement system.

In this paper we characterize hydrogen peroxide present in the sera of chagasic patients, with or without cardiac manifestation, as being responsible for the inhibition of growth of the epimastigotes. The increase of hydrogen peroxide to antiproliferative levels might result from an increase in XO activity in the serum of patients infected with *T. cruzi*.

## 2. Materials and methods

### 2.1. Serum samples

Serum samples were collected from adult patients diagnosed with Chagas' disease (chagasic sera) and from adult individuals never diagnosed with Chagas' disease (control human sera), at the Parasitology Laboratory of "José María Ramos Mejía Hospital", Buenos Aires.

These individuals agreed voluntarily to participate in the study by signing an Informed Consent Form.

According to the clinical manifestations, the chagasic sera were classified as:

- Indeterminate chagasic sera (ICh), from patients with *T. cruzi* specific antibodies without clinical signs of cardiac abnormalities (85 individuals).
- Cardiac chagasic sera (CCh), from patients with recognizable signs and symptoms of chagasic cardiomyopathy (15 individuals).

Non-chagasic sera were classified as:

- Control sera (NCh), obtained from healthy individuals (70 individuals).
- Non-chagasic cardiac (CNCh), from patients with cardiopathies not related to Chagas' disease (12 individuals).

All the sera, chagasic or non-chagasic, were obtained from individuals who had no other chronic inflammatory or autoimmune diseases at the time of the study.

### 2.2. Diagnosis of T. cruzi infection

Patients were diagnosed positive via indirect hemagglutination (IHA) test (Wiener Lab.), indirect immunofluorescence assay (IIFA) and enzyme-linked immunoabsorbent assay, ELISA (Wiener Lab.).

### 2.3. Organisms and media

*T. cruzi* epimastigotes, RA strain were kindly provided by Dr. Estela Lammel and Dr. Elvira Isola from the Department of Parasitology, School of Medicine, Buenos Aires University.

*T. cruzi* epimastigotes were grown at 28 °C in liver infusion/tryptosa (LIT) medium containing the heat-inactivated test serum sample or fetal bovine serum (FBS) for the periods indicated in Section 3. During the incubation period the number of mobile epimastigotes was determined daily in a Neubauer chamber. Mobile parasites were considered viable while sluggish, immobile parasites were excluded. The parasites were counted in triplicate.

Parasite viability was confirmed by Trypan blue assay (Freshney, 1994).

### 2.4. Serum heat inactivation

All the assayed sera were placed in a 56 °C water bath for 30 min in order to destroy heat labile complement proteins.

### 2.5. Antibody depletion

For the removal of specific antibodies, serum samples were adsorbed on sensitized with *T. cruzi* surface antigens erythrocytes (Wiener Lab). Each patient serum was added to the erythrocyte pellet, then mixed and incubated at room temperature for 18 h. After incubation, the mixture was cleared by centrifugation. The procedure was repeated until the ELISA test for *T. cruzi* specific antibodies became negative. Experiments designed to test the antiproliferative effect of immunoglobulin depleted sera used the supernatant.

### 2.6. Serum dialysis

A 3 ml serum sample was placed in a semipermeable nitrocellulose membrane dialysis tube, molecular mass cut off: 6000–8000 and dialyzed with stirring against 15 ml LIT medium at 4 °C for 12 h. Low molecular weight molecules passed through the dialysis membrane while proteins were retained in the tube.

In order to asses the antiproliferative action of the low molecular weight fraction of the serum, the parasite pellet was resuspended in the LIT medium obtained after dialysis which contained 10% of the low molecular weight fraction of the serum. To study the effect of the high molecular weight fraction on the parasite growth the sealed tube was placed into fresh LIT medium and the dialysis was repeated twice. The high molecular weight fraction of the serum was added to the parasite growth medium at 10% concentration, as indicated in Section 3.

### 2.7. Xanthine oxidoreductase activity

Aliquots of sera were assayed in 50 mM sodium carbonate buffer containing 2.4 mM EDTA, 20 μM cytochrome c and 2.4 mM xanthine. The mixtures were incubated at 37 °C for 30 min and 10% trichloroacetic acid was added to each sample in order to stop the reaction. Cytochrome c reduction was monitored at 540 nm using a matched xanthine free negative control sample as blank. The specificity of the detection method was verified using allopurinol, a XOR specific inhibitor. To asses both total XOR (XO plus XDH) and XO activity, the reaction was performed respectively with and without $NAD^+$. In the presence of $NAD^+$, NADH is formed by XDH instead of $H_2O_2$ plus $O_2^-$, and ROS generation elicited by XOR decreased. As a result, the addition of $NAD^+$ to the reaction mixture diminished cytochrome c reduction, and the remaining XOR activity is only due to XO (Muranjan et al., 1997).

### 2.8. Lipid peroxidation and superoxide dismutase activity

The epimastigotes grown in the LIT medium were exposed to each tested serum for 24 h. The cells were then pelleted, homogenized and analyzed for lipid peroxides and superoxide dismutase (SOD) activity.