Trans-sialidase Protein as a Potential Serological Marker for African Trypanosomiasis

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Abstract

Trypanosoma brucei is the etiological agent of African trypanosomiasis responsible for human and animal infections. *T. brucei* is transmitted by infected tsetse flies. There is no vaccine for the disease and drugs available for treatment are inefficient and high toxicity. In this context, it is a priority to find antigenic targets suitable for the development of new diagnostic tools, drugs and vaccines. In this work, we report that mice infected with *T. b. brucei* produce antibodies against *trans*-sialidase recombinant protein (TS). In addition, we also demonstrate that bloodstream *T. b. brucei* express messenger RNA related to the TS gene. Collectively, our data strongly suggest that bloodstream forms of *T. b. brucei* also express the TS gene, that to date was described only in the procyclic forms of the *T. b. brucei*. In conclusion, these results highlight the importance of TS protein as a possible antigen target during infection caused by *T. b. brucei*.

Keywords *Trans*-sialidase · *Trypanosoma brucei* · African trypanosomiasis · Antigenic targets · Serological markers

1 Introduction

Trypanosoma brucei is the etiological agent of African trypanosomiasis (AT) or sleeping sickness in humans and Nagana in animals. AT is a neglected disease caused by *T. brucei*, a protozoan parasite transmitted by infected vector tsetse flies. Two main evolutionary forms characterize the life cycle of *T. brucei*; the procyclic forms present in the vector, and the bloodstream forms present in mammalian

hosts. In the mammalian hosts parasites assume different morphologies and expression patterns [1].

In *T. brucei* bloodstream forms, the parasite's membrane is covered mainly by variant surface glycoproteins (VSG), a family of variable proteins that allows trypanosomes to survive during infection in the mammalian host, causing immune system disorders and symptoms of chronic diseases [2]. In the context of procyclic forms, parasites are coated with procyclin, a membrane protein that replaces VSG from the bloodstream forms of *T. brucei*. VSG coat is constantly changing by antigenic variation, which is a mechanism of immune evasion where VSG molecules are constantly being produced and released in different serotypes [3].

Besides the VSG molecules in bloodstream stages and the procyclin in procyclic forms, *T. brucei* also present other membrane components that interact with the host during infection. One of these components is the *trans*-sialidase protein (TS); an enzyme well described in the procyclic forms of *T. brucei* [4–7]. However, the presence of TS protein is not completely resolved in bloodstream forms of *T. brucei*. Conversely, TS protein has been very well described in *T. cruzi* protozoan, the etiological agent of Chagas disease also denominated of American trypanosomiasis [8–10]. In *T. cruzi*, the TS component is an important toxin, serological maker, and virulence factor during infection, representing a promising target for vaccine development [11–14].

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The TS protein is a membrane-associated enzyme used by trypanosomes to obtain sialic acid from their host, and to incorporate it into their cell-surface glycoproteins [8]. Thus, TS is responsible for transferring sialic acid molecules from the host's cell surface to the acceptor molecules on the parasites' surface, a phenomenon known as sialylation, which occurs in the different stages of development of the parasite, contributing to virulence of the *T. cruzi* [15].

The first isolation and characterization of TS protein was reported in *T. cruzi* [8], followed by an equivalent enzyme from cultured procyclic forms of the African trypanosomes species *T. brucei* and *T. congolense* [6, 16–18]. In contrast to *T. cruzi* parasite, TS has not yet been resolved in bloodstream forms of *T. brucei*, being reported to express in the procyclic forms only. Recently, Ammar et al. [19] have detected TS activity in bloodstream forms of *T. b. gambiense*.

In our previous work, we suggested for the first time the presence of TS protein also in bloodstream *T. b. brucei* [20]. We demonstrated that mice, immunized by DNA vaccine encoding TS gene, produce anti-TS antibodies able to recognize a total crude extract of bloodstream forms of *T. b. brucei*. Interestingly, this immunization process is able to elicit partial protection of mice challenged with infective *T. b. brucei* parasites.

In the present work, we report for the first time that Balb-C mice infected with *T. b. brucei* produced anti-TS antibodies that recognized TS recombinant protein. In addition, *T. b. brucei* bloodstream stages, purified from infected mice, expressed mRNA for TS gene expression. These results support the importance of TS as a possible antigenic serological marker during infection caused by *T. b. brucei* parasites.

2 Results

To date, the literature has reported some inconsistency regarding the presence of TS protein only in the procyclic forms of *T. b. brucei*. In order to verify the presence of this enzyme also in the bloodstreams *T. b. brucei*, bloodstream parasites were purified from blood of infected mice with *T. b. brucei* GVR 35/1.5. Figure 1 shows the purification process of the parasites obtained from blood of infected mice. Whole blood from infected mice was fixed on slides and subsequently stained by Giemsa (Fig. 1a). Then, blood samples were submitted to DEAE-cellulose chromatography and the bloodstreams forms of *T. b. brucei* were obtained, as shown in Fig. 1b. We visualized by light microscopy that the blood cells were eliminated during the chromatographic process, and that the bloodstream forms of *T. b. brucei* were efficiently eluted and purified from the whole blood from infected mice (Fig. 1). Purified parasites were then washed in PBS-glucose and subsequently frozen at -80°C until the time of use. This preparation, containing only bloodstream *T. b. brucei*, was used to obtain genomic DNA, total RNA and crude protein extract of bloodstream forms of *T. b. brucei*.

In order to verify TS gene expression in the bloodstream forms of *T. b. brucei*, parasites purified from blood of infected mice were submitted to extraction and purification of genomic DNA, and total RNA by commercial kit (Bioline—UK). cDNA of bloodstream forms of *T. b. brucei*, obtained by reverse transcriptase-PCR, was then used as a template for PCR amplification of the complete TS gene sequence (2,316 bp) and the first 1,416 bp fragment of the TS gene, here named nTS. Figure 2 shows the illustrative representation of the TS gene sequence, the

Fig. 1 Purification process by DEAE-Sepharose chromatography of bloodstream forms of *T. b. brucei* obtained from infected Balb-C mice. **a** Whole blood from infected mice; **b** bloodstreams forms of the

parasites obtained after chromatography, both slides were fixed and stained by Giemsa's staining (optical microscopy 400x)

Fig. 2 Representative illustration of the cloning strategy of TS gene and purification of genomic DNA and mRNA of bloodstream forms of *T. b. brucei* parasites. **a** Cloning strategy of *trans*-sialidase gene

and nTS sequences. **b** Purification of the genomic DNA and mRNA from bloodstream forms of *T. b. brucei*

TS fragment (Fig. 2a) and the experimental strategy used to demonstrate the presence of TS gene expression in the bloodstream forms of *T. b. brucei* (Fig. 2b).

Afterwards, in order to verify the presence of specific mRNA for TS gene of bloodstream forms of *T. b. brucei*, specific primers were used for amplification of TS gene by conventional and non-quantitative PCR. Simultaneously, the 1,416 bp fragment of TS gene was also amplified. In both cases, cDNA obtained from bloodstream forms of *T. b. brucei* was used as a template for amplification reactions. As shown in Fig. 3, bloodstream forms of *T. b. brucei* have specific mRNA for the TS gene (2,316 bp—lane 3), which is also amplified by PCR for the nTS fragment (1,426 bp—lane 2). As a positive control of PCR, we used specific primers to amplify a stage-specific gene of bloodstream forms of *T. b. brucei*, an Invariant Surface Glycoprotein gene (ISG), about 1,572 bp (lane 1). Figure 3 also shows that ISG and TS genes are not amplified by conventional PCR, when the template consists only of RNA obtained of bloodstream forms of *T. b. brucei* (lanes 4–6). Likewise, both genes are amplified by conventional PCR when the template is genomic DNA obtained from bloodstream forms of *T. b. brucei* (lanes 7–8).

After confirming the presence of the TS gene in the bloodstream forms of *T. b. brucei* by molecular techniques

Fig. 3 Analysis of the presence and gene expression of *trans*-sialidase gene in the bloodstream stages of *T. b. brucei*. Conventional PCR products were subjected to electrophoresis on agarose gel (1% w/v) in TAE buffer, ethidium bromide, and visualized using ultraviolet light. Lanes 1–3: PCR products obtained from cDNA as template; lanes 4–6: PCR product obtained from total RNA as template negative control; lanes 7–8: PCR product obtained from genomic DNA. Both templates were isolated from bloodstream stages of *T. b. brucei*. M molecular marker—bp (Bioline—UK), gDNA genomic DNA

(RT-PCR and conventional PCR), recombinant TS protein was produced in *Escherichia coli* bacteria. Next, in order to use the recombinant TS protein as a possible serological

marker for AT, *T. b. brucei* TS gene was cloned into a gene expression plasmid for bacterial gene expression system (results not shown).

The constructed plasmid DNA, denominated nTSpET28a, was used to transform BL21 (DE3) *E. coli* bacteria and subsequently to express and produce nTS recombinant protein using IPTG induction. Using SDS-PAGE technique, bands corresponding to nTS recombinant protein (about 52 kDa) were obtained (Fig. 4). The nTS recombinant protein, represented by the arrow in the Fig. 4, was purified by removing the band from the SDS-PAGE gel, according to the protocol presented in the session materials and methods (results not shown). Afterwards, purified nTS recombinant protein was subsequently immunidentified by ELISA and immunoblotting (result not shown).

In order to determine the presence of anti-TS antibodies in the mice infected with *T. b. brucei*, blood samples were collected at 0, 5, 14, 21, 25, and 45 days post-infection and sera were obtained. The course of infection was determined by parasitemia in the blood, visualized by optical microscopy (results not shown) and by the presence of total IgG antibodies anti-*T. b. brucei*, determined by ELISA, as shown in Fig. 5. Sera from infected Balb-C mice show an increase in titer of IgG antibodies that specifically recognize a crude extract of bloodstream forms of *T. b. brucei*. We established the experimental model of infection with *T. b. brucei* through the presence of parasites in the blood and the presence of anti-*T. b. brucei* antibodies by ELISA.

Fig. 4 Gene expression of recombinant nTS protein produced in *E. coli* BL21 (DE3) and analysed by SDS-PAGE. Lanes 1 and 2 represent the electrophoretic protein profile of *E. coli* BL21 (DE3) without and with IPTG induction, respectively

Fig. 5 Determination of total IgG anti-*T. b. brucei* antibodies in sera from infected mice with *T. b. brucei* parasites. Bars represent IgG obtained by ELISA from diluted sera (1:200), measured by optical density (absorbance) at different time points post infection. Each bar represents the mean and standard deviation of the absorbance values obtained in duplicate from each group of infected animals (n=3)

In order to determine the presence of anti-TS antibodies in mice experimentally infected with *T. b. brucei*, nTS recombinant protein was used for the determination of anti-nTS antibodies. ELISA was performed to measure the titers of total IgG anti-nTS, as well as the IgG1, IgG2a and IgG3 subtypes (Fig. 6). The titers of IgG anti-nTS antibodies increased with the course of infection caused by *T. b. brucei*, as the same was observed for IgG anti-*T. b. brucei* (Fig. 5). In Fig. 6 it is also possible to observe the profile of anti-nTS subtypes antibodies (IgG1, IgG2a and IgG3).

Fig. 6 Determination of total and subtypes IgGs antibodies against trans-sialidase recombinant protein in sera from infected mice with *T. b. brucei* parasites. The horizontal axis represents days post infection versus optical density (absorbance) obtained by ELISA performed with 1:200 sera dilution. Each bar represents the mean and standard deviation of the absorbance values obtained in duplicate from each group of infected animals (n=3)

3 Discussion

African trypanosomiasis, caused by the flagellated protozoan *T. brucei*, is a neglected tropical disease with great impact on human and animal health. This infectious disease can be found in 36 countries of sub-Saharan African, where there are tsetse flies that transmit the parasite. Although recently the number of new cases of human disease has significantly been reduced [21], AT still affects millions of people and animals, contributing for economic disruption specially in rural communities, representing a important factor responsible for the delay in the development of the affected regions [22, 23].

Control programmes implemented in the context of AT are generally composed by diagnosis and treatment of new clinical cases. However, drugs used in the treatment against *T. brucei* are ineffective and present too much toxicity; diagnosis and treatment of the disease is complex and requires specifically skilled staff and laboratory diagnostic methods have low specificity and sensitivity. Considering the difficulties that both diagnostic and treatment present, it is necessary to identify potential new targets that can be used for the development of new drugs and new diagnostic markers for the control of AT.

The main purpose of this work is to use *trans*-sialidase protein (TS) as a possible serological marker in infections caused by *T. brucei*, and to demonstrate the presence of the TS in the bloodstream stages of *T. b. brucei*. It is important to highlight that, until now, the TS enzyme has only been described in the procyclic forms of *T. brucei*. Thus, we could enhance the use of TS as a possible serological marker in infections caused by *T. brucei*.

The expression and presence of TS protein in bloodstream forms of *T. brucei* is controversial and has only been described in the procyclic form, a situation in which the parasites replicate in the invertebrate host [4–6]. Procyclins proteins cover the procyclic forms of *T. brucei*. Procyclins have an uncommon GPI anchor that, unlike the GPI anchor of VSGs, is composed by poly-*N*-acetyl-lactosamine repeats capped by sialic acid residues [4, 5]. Nakatani et al. [24] suggest that TS protein plays a role in the sialic acid modification of the procyclic stage *T. brucei* cell surface.

The sialic acid contributes for the biological properties of cell surface. In *T. cruzi* parasites, the etiological agent of Chagas disease, the sialic acid has been implicated in cell–cell interactions and T-cell activation, a key for their survival in the blood [25]. It has been suggested that sialylated molecules are involved in the invasion of mammalian host cells and in the protection from complement lysis [5]. Furthermore, Trypanosomes, that do not

acquire sialic acids due to a defect in the production of the sialylated GPI anchors, do not survive in the intestine of the vector. Thus, the sialic acid present on cell surface appears to protect parasites from the environment in the midgut of the tsetse fly [26].

In this work we demonstrated that bloodstream forms of *T. b. brucei*, purified from blood of infected mice, express the TS protein. This finding was obtained using cDNA produced from RNA extracted from bloodstream forms of *T. b. brucei* purified from infected mice. Interestingly, the presence of the TS protein in the bloodstream forms of *T. b. brucei* was demonstrated indirectly through the production of anti-TS IgG antibodies in *T. b. brucei* infected mice, and by immunoblotting (results not shown). However, the biological function and molecular characterization of the TS protein from bloodstream stages of *T. b. brucei* are not widely known and investigated.

In a previous work, we hypothesized that TS could be expressed in the bloodstream stages of *T. b. brucei*. Our work has showed that mice vaccinated with plasmid DNA encoding the 5'-terminal region of the *trans*-sialidase (nTS) gene of *T. b. brucei* were able to produce IgG antibodies that bind to the bloodstream forms of *T. b. brucei*, and consequently recognize the recombinant nTS protein [20]. These findings allowed us to suggest that TS protein (or unlikely a TSA-like cross-reactive protein) was also expressed in the bloodstream forms of *T. b. brucei*, in similarity to what happens with *T. cruzi* [8, 9].

Our results suggest for the first time that TS protein is expressed in the circulating bloodstream forms of *T. b. brucei*, thus constituting a potential antigenic target, up until now not considered. Nevertheless, a more detailed study is required regarding the levels of mRNA expression in the bloodstream forms of *T. b. brucei*. In this study, we report a preliminary reverse transcriptase-PCR (RT-PCR) study to analyse the presence of TS mRNA. However, a quantitative PCR will be required to determine TS mRNA levels in these bloodstreams forms of *T. b. brucei*.

In this work, we believe that TS bloodstream protein may be a potential antigen target due to its ability to generate antibodies during the course of infection caused by *T. b. brucei*, and may be an important antigen, responsible for the induction of the immune response during the parasite–host interaction. It is also important to highlight the type of anti-TS antibodies generated during AT, preferably Th1-type antibodies (IgG2a and IgG3). This pattern of immune response is expected since *T. b. brucei* stimulates a Th1 immune response with increased titres of IgG2a. With this finding a whole new need is emerging: to investigate and understand the biological role of TS protein in the bloodstream stages of *T. b. brucei*.

4 Materials and Methods

4.1 Parasites and Mice

Strain *T. b. brucei* GVR 35/1.5 was used for experimental infection of AT in murine model [27]. Animals used in this work were female Balb/C mice between 5 and 8 weeks old, obtained from Institute of Hygiene and Tropical Medicine, Lisbon—Portugal.

4.2 Experimental Model of *T. b. brucei*

For experimental AT infection, mice were inoculated intraperitoneally with approximately 500 parasites, prepared by dilution of the infected blood with PBS-glucose 20 mM pH 7.4. The murine model of infection with *T. b. brucei* is extensively used in our laboratory [19, 28, 29]. Serum samples from *T. b. brucei*-infected mice were obtained at 0, 5, 14, 21, 25 and 45 days post-infection. All sera were stored at -20°C until use. Next, serum samples were used for the detection of anti-*T. b. brucei* and anti-TS protein antibodies by ELISA.

4.3 Gene Cloning and Protein Expression of Trans-sialidase

Plasmid DNA (nTSpVAX1), previously constructed by Silva et al. [19], was used as template for nTS gene amplification by PCR. The reaction was performed with 10 ng of plasmid nTSpVAX1, 100 pmol of primers, 0.5 mM of mix dNTPs (Bioline-Germany), 3 mM MgCl_2 , 5 U BiotaqTM DNA polymerase (Bioline-Germany), and NH_4 buffer (Bioline-Germany). Primers sense (5'ATTATAGCTAGCATGGAG GAACTCCACCAAC'3) and anti-sense (5'TAATCCCTT AAGTCAGTGCAGACAATAA'3) were designed to amplify about 1416 bp from the trans-sialidase gene [5], here referred as nTS gene. The PCR amplicon was enzymatically digested by NheI and EcoRI endonucleases (Fermentas-USA) and cloned in the pET28a plasmid (Novagen - USA). The constructed plasmid (nTSpET28a) was submitted to restriction analysis and sequencing (data not shown).

For gene expression of the recombinant nTSA protein, competent cells *E. coli* BL21 (DE3) were transformed with nTSpET28a by heat shock. The cells were selected with kanamycin antibiotic (Bioline—UK) and expanded with agitation at 350 rpm in LB medium at 37°C up to approximately 0.8–1 optical density absorbance (600 nm). The nTS gene expression was induced by adding 0.5 mM of isopropylb-D-1-thiogalactopyranoside (IPTG) for 18 h at 37°C and 350-rpm agitation. Cells were then centrifuged, pelleted and suspended in buffer saline (PBS pH 7.4). SDS-sample buffer

(v/v) was added, and this mixture was reduced for 5 min in a boiling water bath. Samples were then submitted to sodium SDS-PAGE. The partial purification of nTS recombinant protein was preceded according to established protocols [30]. The partial purified proteins were quantified and visualised by SDS-PAGE stained with Coomassie blue R-250 to confirm the presence of the partial purified protein. The partial purified nTS protein was used as the antigen source for the serological assay (ELISA) and immunoblotting.

4.4 Identification of the Trans-sialidase Expression in the Bloodstream *T. b. brucei*

Blood samples from *T. b. brucei*-infected mice were collected and used for the RNA extraction, according to RNA isolation Kit, (Bioline, UK). Concentration of total RNA was determined by NanoDrop 1000 (Thermo Scientific). The cDNA synthesis was performed according to the cDNA Synthesis Kit (Bioline, UK). Complementary DNA was used as template for nTS gene amplification by polymerase chain reaction (PCR) assay. The reaction was performed with 100 ng of cDNA, 100 pmol of primers, 0.5 mM of mix dNTPs (Bioline-Germany), 3.0 mM MgCl_2 , 5.0 U BiotaqTM DNA polymerase (Bioline-Germany), and NH_4 buffer (Bioline-Germany). The same specific primers, previously described above, were used to amplify the first 1,416 bp from the TS gene (designated nTSA gene). Reaction mixtures were incubated in a thermocycler (MJ-Mini Bio-Rad-Singapore) at 94°C for 2 min, followed by 40 cycles of 30 s at 94°C , 1 min and 30 s at 60°C , and 2 min at 72°C . After the final cycle, reaction mixtures were cooled down at 4°C . The amplified product was examined by performing a 1% agarose gel electrophoresis ran at 100 V in TAE buffer (40 mM Tris base, 20 mM acetic acid, pH 8.0, 1 mM EDTA), stained with ethidium bromide.

4.5 Determination of Anti-*T. b. brucei* and Anti-trans-sialidase Antibodies by Enzyme-Linked Immunosorbent Assay

Total protein of bloodstream forms of *T. b. brucei* and partially purified nTS recombinant protein were used to adsorb 96-well micro-plates to perform indirect enzyme-linked immunosorbent assays (ELISA), using sera from *T. b. brucei*-infected mice. Total protein of bloodstream forms of *T. b. brucei* was obtained from heparinized total blood collected from infected mice. The parasites were purified using a gravity-operated DEAE-cellulose chromatography column described by Lanham and Godfrey [31].

Total serum immunoglobulin G (IgG) specific for *T. b. brucei* and for nTSA recombinant protein was measured. Additionally, IgG1, IgG2a and IgG 3 antibodies were also determined. All sera from infected mice were assayed in duplicate

and specific antibodies measured at 490 nm. Previously, 96-well micro-plates (BD Falcon—USA) were coated with 100 ng/well of partial purified nTSA in carbonate buffer pH 8.5 (overnight at 4 °C). The micro-plates were washed three times with PBS-0.05% Tween20 (Promega—USA) and blocked for one hour at room temperature with 1% (w/v) bovine serum albumin solution in PBS. After three washes, serial dilution of infected mice sera were added for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG (diluted 1:4000 v/v—Sigma Chemical Co—USA), rat anti-mouse IgG1 (500 ng/mL—AbDserotec—UK), rat anti-mouse IgG2a (250 ng/mL—AbDserotec—UK) and rat anti-mouse IgG3 (diluted 1:4000 v/v—AbDserotec—UK). The HRP-conjugated antibodies were incubated for 30 min with a substrate solution (10 mL of citrate buffer pH 5.0 with 10 mg of OPD and 10 µL of hydrogen peroxide 30%—Sigma—USA). Then, sulphuric acid 4N was used as stop solution and absorbance was determined 490 nm by spectrometry. This assay was developed according to the protocol developed by [20].

5 Conclusions

We concluded that during the course of infection caused by *T. b. brucei*, mice show increased titers of antibodies specific to TS protein, represented herein as nTS recombinant protein. These data open the opportunity to evaluate the biological function of TS protein during the parasite-host interaction process—a serological marker and a potential target for vaccine development in the context of AT.

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Author Contributions Conceived and designed the experiments: AFT and MSS; Performed the experiments: AFT, JGP, and SPA; Analysed the data: AFT, JGP, SPA, and MSS; Wrote the paper: AFT and MSS. All authors read and approved the final version of the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethics Approval Murine model was performed in accordance with existing legislation in Portugal and in accordance of the Ethical Committee at Institute of Hygiene and Tropical Medicine, Lisbon—Portugal.

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