CHARACTERIZATION OF A SOLUBLE FACTOR RELEASED BY *TRYPANOSOMA BRUCEI* SP THAT INDUCED ARGINASE ACTIVITY IN MACROPHAGES

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ABSTRACT

Extracellular trypanosomes can modulate the immune cell function through parasite soluble factor (PSF) that induce arginase activity. In this report, we aimed to identify the soluble factor that induce arginase in macrophages. Trypanosomes were obtained from infected mice , the purification is performed parasites on a DEAE - cellulose column. Parasites were cultured in minimal medium at 37 ° C with 5% CO2 for 2 hours. The supernatant containing the PSF is used to activate the macrophage arginase .

Macrophage arginase was induced by *Trypanosoma brucei gambiense* and by their PSF *in vitro*. We also identified by electrophoresis and Western blot a 120 kDa protein as the main arginase-inducing factor in the PSF. This factor consists in a dimer of 60 KDa protein, which do not stimulate the induction of arginase individually. The identification of a such marker of immune dysregulation observed during Human African trypanosomiasis may help in the amelioration of diagnosis and even treatment with study of the signaling pathway involving these proteins.

Keywords: PSF, arginase induction, macrophages, *Trypanosome brucei sp, in vitro*.

RÉSUMÉ

Les Trypanosomes extracellulaires peuvent moduler la fonction des cellules immunitaires à travers les facteurs solubles parasite (PSF) qui induisent l'activité de l'arginase. Dans ce travail, nous avons cherché à identifier le facteur soluble qui induit l'arginase macrophagique. Les trypanosomes ont été obtenus à partir de souris infectées, la purification des parasites est faite sur une colonne de DEAE-cellulose. Les parasites ont été cultivés dans un milieu minimum à 37°C contenant 5% de CO_2 pendant 2 heures. Le surnageant contenant le PSF est utilisé pour activer l'arginase macrophagique. L'arginase macrophagique a été induite par *Trypanosoma brucei gambiense* et leur PSF *in vitro*. Nous avons également identifié par électrophorèse et western blot une protéine à 120 kDa comme le principal facteur inducteur de l'arginase dans le PSF. Ce facteur consiste en un dimère de 60 kDa , qui ne stimulent pas l'induction de l' arginase dans sa forme monomère. L'identification d'un tel marqueur de dysrégulation immunitaire observée au cours de la trypanosomiase humaine africaine peut aider à l'amélioration du diagnostic et même du traitement par étude de la voie de signalisation impliquant ces protéines.

Mots clées: PSF, induction arginase, macrophages, *Trypanosome brucei sp, in vitro*.

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INTRODUCTION

Trypanosomes of the brucei group in the extracellular fluid, are the cause of human African trypanosomiasis (HAT) known as sleeping sickness. This disease represents a public health problem in the rural areas of some of Africa region. It is also African animal trypanosomiasis (AAT) known as Nagana and cause to be one of the greatest constraints to livestock production (Connor 2009).

Diagnosis, monitoring and prognosis remain difficult because of the nonspecific disease progression. The clinical signs of HAT, especially in the early stages, are difficult to distinguish to the other infectious diseases. Progresses through an early stage when parasites proliferate in the haemo-lymphatic system causing a febrile illness, followed by a second or late stage of disease in which parasites invade the central nervous system (CNS) causing meningo encephalitis (Malvy 2011).

Initial screening of patients involves indirect diagnostic techniques, the most widely used based on serological test and known as the Card Agglutination Test for Trypanosomiasis (CATT) (Chappuis *et al*., 2005). The CATT is based on the agglutination by serum antibodies of lyophilized bloodstream forms of *T. b. gambiense* expressing variant surface glycoprotein type LiTat1.3, which is expressed widely in *T. b. gambiense* isolated. This must be followed by parasitological diagnosis, which is laborious, may require high level techniques due to low parasitaemia, and must be carried out by skilled microscopists. As extracellular trypanosomes proliferate and live freely in the bloodstream and lymphatics, they are constantly exposed to the immune system (Cnops et

al., 2014). African trypanosomes have acquired various mechanisms to manipulate and control the host immune response, evading effective elimination. The main immunomodulating mechanism of antigenic variation by clone-specific singular VSG expression combined with switching from VSG to another. Other immunomodulating pathways were also highlighted in trypanosomiasis. *In vitro*, parasite bloodstream forms or soluble factors (or secretome) from both strains induced macrophage arginase as a function of their virulence.

Arginase expression, a hallmark of macrophage alternative activation pathway, increases trypanosome bloodstream forms development (Holzmuller et *al*., 2008). Macrophages are important effectors cells involved in various phases of immune responses, such as phagocytosis, antigen presentation, and secretion of bioactive molecules (Garrido *et al.,* 2011). However, pathogens have developed several strategies to circumvent protective responses of host cells. When trypanosomiasis parasites develop a mechanism to escape the effect trypanostatic/trypanocidal nitric oxide (NO) pathway activation of arginase (Vincendeau et *al.*, 1992)**.** The arginase and NO Synthase (NOS) consume the same substrate which is L-arginine (Duleu et *al*., 2004). Depletion of the substrate induced by arginase removes the cytotoxic effects of NO of macrophage (Gobert et *al*., 2000) and increases the production of polyamine from ornithine (urea cycle). Polyamines are essential for proliferation of trypanosomes involved in DNA synthesis, cell differentiation and synthesis of trypanothione.

The activation of arginase by parasites is due to soluble excreted/secreted factors. The identification of soluble factors activating on macrophages deserves investigation in macrophages. These factors are obtained from purified parasites from mouse blood; its components are analyzed in terms of physico-chemical and biological. Following these studies, we identified a set of proteins of molecular weight (MW) of 120 kDa active way of arginase, and degradation products of around 60 kDa MW. This study aims to all the components of the PSF by electrophoresis to separate by chromatography gel filtration and affinity to test their individual abilities to activate arginase.

Diagnosis of human African trypanosomiasis (HAT) remains a challenge both for active screening, which is critical in control of the disease and in the point-of-care scenario where early and accurate diagnosis is essential. In this paper we characterize the arginase inducing factor contained in the secretion of the parasite. Thus, we hypothesize that during *T. brucei* infection, fraction may contact with macrophage arginase activity and promoting the intracellular growth of parasites. The detection of this concept is a therapeutic target that may allow interaction with parasite development. .

MATERIALS AND METHODS

Mice

Female Swiss mice (8 to 12 weeks old) were purchased from Breeding Center DEPRE (Saint Doulchard, France).

Parasites and parasite solubles factors (PSF)

Trypanosoma brucei gambiense (T. brucei gambiense) "Feo" (ITMAP/1893), responsible for the chronic form of the Human African Trypanosomosis (HAT). *Trypanosoma brucei brucei (T. brucei brucei)* (Antat 1.9), parasite of animals. Trypanosomes were purified DEAE-cellulose. Parasite soluble factors were prepared in a secretion medium according to a method previously described (Holzmuller *et al.*, 2008). Purified parasites (2×10^8/ml) were incubated in medium for 2 hours at 37°C. Supernatant containing ESF was separated from trypanosomes by centrifugation (1,000 g, 10 min, 4°C) and filtered using a 0.22 µm.

Macrophage culture

Macrophages were obtained by washing the peritoneal cavity of mice with 4 ml HBSS. Cells were plated onto 96-well polystyrene plates for 1 h at 37°C under 5% CO_2 atmosphere. Cultured macrophages were additioned PSF for 72 h at 37°C under 5% CO_2 atmosphere.

Determination of the macrophage arginase activity

The macrophages arginase activity is determined by using methods previously described by Corraliza et *al*., (1994). Briefly, 10^5 cells were lysed with 0.5 ml 0.1% Triton X-100. After 30 min, 0.5 ml of 25 mMTris-HC1 containing 5 mM MnC12, pH 7.4 was added. The enzyme was then activated by heating for 10 min at 56°C. Arginine hydrolysis was carried out by incubating 25 µ1 of the activated lysate with 25 µl of 0.5 M arginine, *p*H 9.7, at 37°C for 60 min. The reaction was stopped with 400 µ1 of H_2SO_4 (96%): H_3P0_4 (85%) : H_20 (1: 3 :7 v/v). Urea concentration was measured at 540 nm after addition of 25 µ1 of 9% a-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 100°C for 45 min.

Immunoaffinity chromatography

2C12 monoclonal antibody was covalently coupled to CNBr-activated Sepharose 4B Flast flow particles and packed into a column. The immobilized antibody was specifically retained the antigen from a solution PSF passed through the support. Bound antigen were eluted by 0,2 M glycine –HCL pH 2.5 and dialyse in PBS (Semballa et *al*., 2003).

Electrophoresis and Western Blot

To separate protein PSF based on size as first described

by Laemmli (1970). PSF were separated by SDS-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and stained with Coomassie blue G250.

Western blot assays were carried out by transferring the proteins in the second polyacrylamide gel into nitrocellulose membranes. Membrane was incubated with the 2C12 diluted 1:1000 overnight at 4°C.

After three washes, membrane was incubated for 2 hours at 37°C with biotinylated anti-mouse Immunoglobulin (Ig) diluted 1:1000, then 2 hours at room temperature with streptavidin-HRP (Horse Radish Peroxidase) diluted 1:10,000.

After another set of three washes, membrane was immersed in a developer solution (TBS containing: 50 mg of DAB (3,3-diaminobenzidine), 150 mg of 4-chloro-1-naphthol, 50 µl of H_2O_2 diluted in methanol. The reaction was stopped by washing the membrane with distilled water (Pettegrew et *al*., 2009).

RESULTS

Determination of arginase activity in murine macrophages activated

A 5 fold-increase in arginase activity was observed in murine macrophages infected *ex vivo* with *T. b. gambiense* when compared to uninfected cells (Fig. 1A). The induction of arginase was also observed in cells treated with *T. b. gambiense*-derived PSF in a concentration-dependent manner (Fig. 1B). Similar results were obtained with live or PSF-derived from *T. b. brucei* (data not shown). For the following experiments, we worked only with the PSF of *T. b. gambiense*. *T.b.gambiense* releases factors able to trigger arginase activation in macrophages.

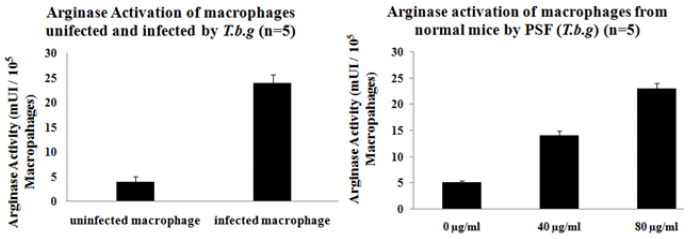

Figure 1: *a shows the arginase activity of mouse macrophages infected with T. b. gambiense, there is also a good activation of the enzyme. B: shows the activation of arginase macrophages from normal mice by PSF.*

Inhibition of arginase stimulation by Antibody (Ab) 2C12

We have developed an Ab that possesses a strong affinity for PSF fraction. This Ab was able to block PSF-induced arginase activity in peritoneal macrophages. This was observed when cells were stimulated with 40 or 80 µg/ml PSF (Fig 2).

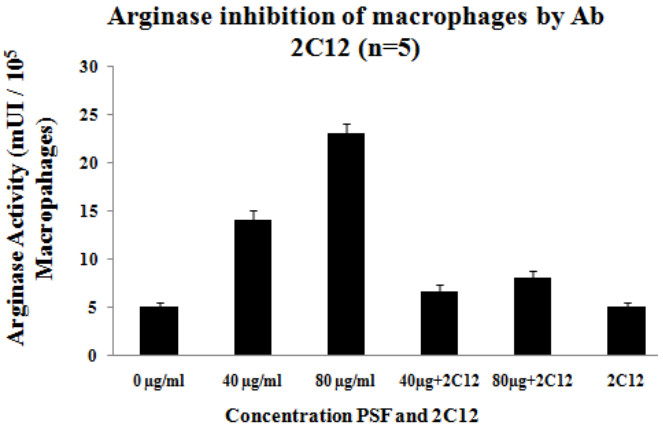

Figure 2: *shows an inhibition of arginase activity, 2C12 dependent.*

Purification of the parasitic soluble factors inducing the path of arginase

We first analyzed 2C12 binding on sepharose 4 fast flow B CNBr by a spectrophotometric analysis. We found that 80% of 2C12 was fixed on the column. Moreover, we observed that 25 % of the total protein content of PSF was bound by the 2C12 column.

Our results show that the FA fraction absorbed contains several bands of molecular weight 60 kDa (Fig. 3). However, two major proteins, with a 60 kDa and 120 kDa molecular weight, were identified on the gel. Both proteins were found to be recognized by the 2C12 Ab (Fig. 4). Then,

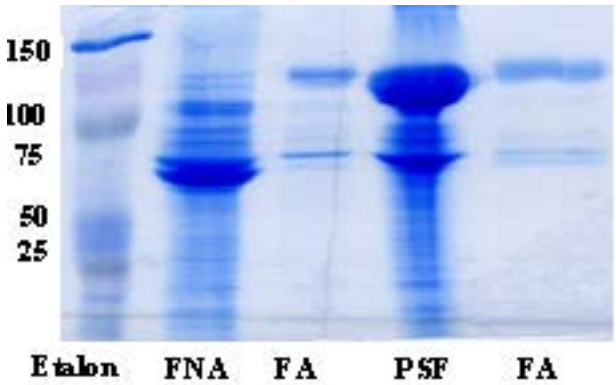

Figure 3: *shows that the fraction absorbed (FA) contains several bands of molecular weight between 120 to 130 kDa and between 60 to 65 kDa, suggesting that either the presence of a protein in two or more units, or the presence of two different proteins.*

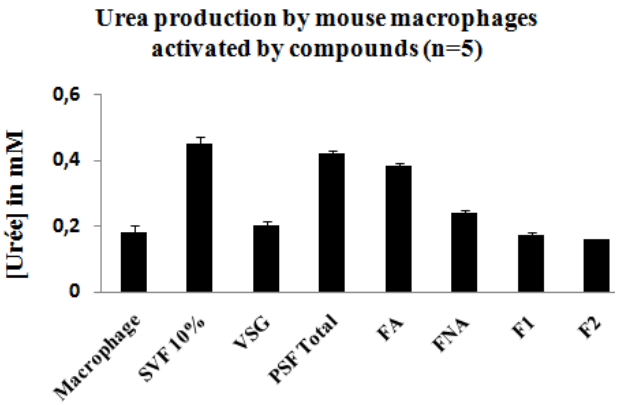

Figure 4 : *Activation arginase of murine macrophages by: PSF, Variant surface Glycoprotein (VSG), fraction A (FA), fraction NA (FNA)*

we dropped the sample absorbed (FA) at 4°C for two weeks then we realized electrophoresis and Western blotting with Ab 2C12. We found no band at 120 kDa but increased expression of 60 kDa band that were recognized by 2C12 (data not shown).

Analysis of proteins PSF inducing arginase

Our results indicated that PSF, FA, and SVF used as positive control were able to induce arginase in macrophages, whereas arginase activity was not modified above untreated cells in response to FNA, VSG, and both fraction used as negative control, i.e. fractions F1 and F2 (Fig. 5).

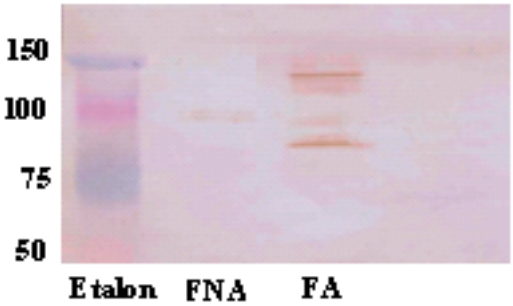

Figure 5: *Western blot of these fractions (FNA, FA) revealed by Ab 2C12*

DISCUSSION

Because trypanosomes are extracellular parasites, we sought to understand how they were able to stimulate the induction of arginase via their secretion of soluble factors. These factors were purified by chromatography affinity using a monoclonal Ab (2C12) developed in our laboratory. We found that arginase activity was induced by the fraction immunopurifed using 2C12. This fraction is mainly characterized by two major proteins with 60 and 120 kDa in Laemli condition. Both proteins are recognized by 2C12 Ab, suggesting that only one active protein with 60 kDa subunits has been identified.

The monoclonal Ab (2C12) we identified by affinity chromatography and western blot, a family of proteins directly involved in the activation of arginase. These proteins are temperature sensitive and inactivated by proteolysis enzymes. The dimeric form of the protein remains in the presence of SDS and dissociated in the presence of 2-mercaptoethanol; proving that the connection is not low. The protein is fragile and does not induce arginase in its monomeric form. The monomeric form contains epitopes which can be used in ELISA for the detection of the signature of trypanosomes in the serum of patients. Dimeric form conformation of the active fraction and its fragility suggests that the induction of this pathway is subject to many environmental parameters.

Many cytokines are also involved in the activation of the arginase pathway. TGF-β is a potent activator of the path of arginase (Gong et *al*., 2012; Boutard et *al*., 1995), and many studies have evidenced the role of TGF-β in the survival of *Leishmania* and *Trypanosoma cruzi* (Anderson et *al.*, 2008; Araújo-Jorge et *al*., 2012; Weber et *al.*, 2005). Inversely, our results demonstrate that African trypanosomes directly induce arginase by their soluble factors. It has been shown that *Trypanosoma cruzi* antigen cruzipain-macrophage interaction could modulate the immune response against *T. cruzi* though the induction of preferential arginase metabolic pathway (Garrido et *al*., 2011).

These results suggest that PSF fraction products regulate host immune response at the macrophage level inhibition of pro-inflammatory cytokines production and induction of macrophage toward the alternative phenotype, which maybe important for worm survival and host health. This mechanism is a parasite escape mechanism, the identification of the receptor (first messenger) macrophage this parasitic factor may lead to the characterization of the signaling pathway leading to the development of inhibitors for therapeutic purposes, provide new potential molecular targets for improved field diagnosis and is important, because they are the promising strategy to control and the prevent parasite infection.

Identification of the fraction will give better insight into the parasite-macrophage interaction. Inhibition of the fraction of interest of PSF by monoclonal antibodies and inhibition of macrophage receptor is a diagram of possible co- therapy. The dose -response relationship of the active fraction shows that the parasite secretory capacity determines the severity of the disease, the virulence and the pathogenicity.

The diagnosis of HAT in endemic foci is mainly based on the detection of antibodies in all subjects by agglutination of trypanosomes methods (CATT). The CATT is based on the agglutination by serum antibodies of lyophilized bloodstream forms of *T. b. gambiense* expressing variant surface glycoprotein type LiTat1.3. (Van Nieuwenhove et *al*., 2011). However, trypanosomes carry a variable surface glycoprotein (VSG), which explains the possibility of false negative results. We have reported that serum arginase is a biomarker of the disease, and normalization of serum arginase may be a marker of treatment efficacy (Nzoumbou-Boko et *al*., 2013). In this context, the present study may open a novel pathway for improving efficacy of HAT diagnosis. We propose that the arginase-inducing factor contained in the PSF and/or the Ab against this factor could be a novel marker of early detection of phase 1 in sleeping sickness in endemic areas.

CONCLUSION

During co-evolution within their hosts, trypanosomes have conserved a mechanism favoring their multiplication and diverting host immune response. The PSF of *T. b. gambiense* or *T. b. brucei* and the monoclonal Ab 2C12 allowed us to highlight the soluble factors excreted in the activation of arginase. Secreted fraction play a major

role in disease development processes and host-pathogen interactions, and usually determine the virulence parasite. We provide here evidence that 2C12 inhibited arginase-inducing activity of PSF, suggesting that arginase induction is a conserved escape mechanism devised by trypanosomes. This is a high MW protein, recognized by 2C12, consisting of two subunits, which individually do not activate the arginase. The identification of this protein by Maldi-Tof analysis deserves now further experiments and may favor the improvement of HAT diagnosis.

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