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Identification of a tryptophan-like epitope borne by the variable surface glycoprotein (VSG) of African trypanosomes

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Abstract

Antibodies (Ab) directed against a tryptophan-like epitope (WE) were previously detected in patients with human African trypanosomiasis (HAT). We investigated whether or not these Ab resulted from immunization against trypanosome antigen(s) expressing a WE. By Western blotting, we identified an antigen having an apparent molecular weight ranging from 60 to 65 kDa, recognized by purified rabbit anti-WE Ab. This antigen, present in trypomastigote forms, was absent in procyclic forms and *Trypanosoma cruzi* trypomastigotes. Using purified variable surface glycoproteins (VSG) from various trypanosomes, we showed that VSG was the parasite antigen recognized by these rabbit Ab. Anti-WE and anti-VSG Ab were purified from HAT sera by affinity chromatography. Immunoreactivity of purified antibodies eluted from affinity columns and of depleted fractions showed that WE was one of the epitopes borne by VSG. These data underline the existence of an invariant WE in the structure of VSG from several species of African trypanosomes.
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Index Descriptors and Abbreviations: *Trypanosoma brucei*; *Trypanosoma brucei gambiense*; *Trypanosoma evansi*; *Trypanosoma cruzi*; *Trypanosoma musculi*; Trypanosome; L-tryptophan (W); VSG, Variable surface glycoprotein; African trypanosomiasis; Antibodies; Epitope; Immunoblotting; HAT, human African trypanosomiasis; WE, tryptophan-like epitope; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay

1. Introduction

Trypanosomes of the *brucei* group are the causative agents of infections in man and animals. *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* cause human African trypanosomiasis (HAT), also called sleeping sickness (Dumas and Bouteille, 1997). *Trypanosoma brucei brucei* causes nagana, a disease in cattle and small ruminants. The closely related *Trypanosoma evansi* infects camels, horses, water buffaloes and other species. These diseases are characterized by relapsing parasitemia, associated with the expression of antigenic variants caused by rapid switching of the major variable surface glycoprotein (VSG) (Turner, 1984). VSG form a dense coat on the surface of the parasite. VSG which have an apparent molecular weight of about 60–65 kDa are highly immunogenic and cause the production of VSG-specific Ab (Cross et al., 1984; Pays, 2006). Soluble forms of VSG can be obtained after activation of an *endogenous enzyme* which cleaves the hydrophobic moiety of the membrane form (Cardoso de Almeida and Turner, 1983; Fergusson et al., 1985a,b; Johnson and Cross, 1979).

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Human African trypanosomiasis (HAT) is characterized by the appearance of neurological disorders. In the late stage of the disease, the daytime somnolence and nocturnal insomnia are features of the disturbed circadian rhythm (Buguet et al., 1993). The mechanisms of this pathology are not fully elucidated and could involve the direct role of parasites or of a parasite-derived product such as tryptophol and/or autoimmune phenomenons (Stibbs and Seed, 1973; Stibbs and Curtis, 1987). Sleeping sickness is marked by an important autoantibody (auto-Ab) production. These auto-Ab are produced against a wide range of antigens including smooth and striated muscles as well as single-stranded deoxyribonucleic acid (Kazyumba et al., 1986). Other auto-Ab are directed against central nervous system (CNS) constituents, including anti-galactocerebrosides (Amevigbe et al., 1992), anti-myelin basic protein (Hunter et al., 1992), anti-neurofilaments (Ayed et al., 1997), and, anti-myelin protein preparations (Asonganyi et al., 1989). Very recently autoantibodies (anti-Sm, anti-Ku, and anti-ribosomal Po protein) considered as specific of systemic lupus erythematosus, have been reported in HAT (Guillaume et al., 2003; Kirrstetter et al., 2004). These auto-Ab might result from polyclonal B cell activation and/or molecular mimicry between trypanosomes and host tissues.

L-Tryptophan is an essential amino acid, which is a precursor of serotonin, involved in sleep regulation. High levels of antibodies (Ab) directed against a tryptophan-like epitope (WE) have been found in sera of patients with sleeping sickness, and were not detected in other diseases such as malaria, HIV-infections, Parkinson and Chagas diseases (Okomo-Assoumou et al., 1995a). These Ab were correlated with the severity of the disease and might be involved in the pathophysiology of HAT (Schultzberg et al., 1988).

In the present report, we investigated the presence of a WE in salivarian trypanosome components. By Western blotting we find the presence of this epitope on several VSG from various trypomastigote forms of salivarian trypanosomes. This epitope was unrelated with the cross-reacting determinant (CRD) associated with the GPI anchor. Immunoreactivities of affinity-purified and affinity-depleted antibodies indicated that WE was one of the epitopes borne by VSG.

2. Materials and methods

2.1. Mice and rabbits

Female Swiss mice (8 to 12 weeks old) and outbred rabbits (15 weeks old) were purchased from Iffa Credo (L'Arbresle, France). Animals were housed and handled according to University approved guidelines.

2.2. Parasites

2.2.1. Bloodstream trypanosomes

Mice were injected intraperitoneally with 10^4 parasites without any treatment. Mice with high parasitemia were bled by cardiac puncture (Vincendeau et al., 1992). Trypanosomes were then purified by DEAE–cellulose chromatography as described by Lanham (1968). Different species of trypanosomes were used (Noireau et al., 1989; Truc et al., 1991): (i) 3 strains of *T. b. gambiense* subspecies: "Féo" (ITMAP/1893), "OK" (ITMAP/1841), "Biyamina" (MHOM/SD/82), isolated respectively from patients living in Togo, Congo and Sudan (Lapierre and Coste, 1963; Daudet et al., 1994), (ii) *T. b. brucei* (AnTat 1.1 and AnTat 1.9) derived from a bushbuck kindly provided by N. Van Meirvenne and D. Le Ray from the Institute of Tropical Medicine in Antwerp, and (iii) *Trypanosoma musculi* (Partinico II) obtained from the London School of Hygiene and Tropical Medicine, London, England (Krampritz, 1969). These parasites were adapted and maintained *in vivo* by passages in normal mice. Stocks of these parasites were kept frozen in liquid nitrogen as stabilates.

2.2.2. Procyclic trypanosomes

Procyclic forms were cultured at 27 °C according to the method of Cunningham (1997). They were derived from *T. b. gambiense*: Mabaya (MHOM/CG/89/MABAYA), Murraz 15 (MHOM/BF/80/MURRAZ15), Dal 972 (MHOM/CL/86/DAL972), and NGB (MHOM/CG/89/NGABELLA), and from *T. b. brucei*: C126 (MPAM/TZ/71/C126) and SH86 (MOVS/86/SH86).

2.2.3. Trypomastigotes of Trypanosoma cruzi

Differentiation of *T. cruzi* was performed in vitro according a method described by Contreras et al. (1985). Briefly, 5×10^6 epimastigote forms of *T. cruzi* (strain TC SO4, clone1), grown in a liver infusion tryptose (LIT) medium, were transformed into trypomastigote forms by a 6 day-incubation at 27 °C in artificial triatomine urine (190 mM NaCl, 8 mM phosphate buffer, pH 6, 17 mM KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$) supplemented with 2.5% sodium bicarbonate 10 mM L-proline. This method enabled us to obtain a high yield (more than 90%) of metacyclic trypomastigote forms.

2.3. Trypanosome antigen preparation

Purified bloodstream trypanosomes suspended at 10^8 cells/ml in NaCl (150 mM) containing glucose (30 mM) and KCl (5 mM) were sonicated (whole trypanosome antigen). Soluble antigens released from parasites were obtained as previously described (Bate et al., 1989; Daulouède et al., 2001). Supernatants were passed through a 0.2 μm pore-size membrane filter (Millipore) and stored at −80 °C until used. Protein concentration was measured by the Bradford method (Bradford, 1976).

2.4. VSG preparation

VSG from *T. b. gambiense* (Féo, LiTat 1.3, LiTat 1.5, and LiTat 1.6), from *T. b. brucei* (AnTat 1.1) and from *T. evansi* (RoTat 1.2) were prepared following a standard

protocol in use at the Department of Parasitology (Institute of Tropical Medicine, Antwerp) for the production of diagnostic VSG antigen (Boutignon et al., 1990; Büscher et al., 1991).

CRD-negative VSG from *T. b. brucei* AnTat 1.1 (ITG) was prepared by aqueous hydrofluoric acid treatment of purified soluble VSG and purity was checked by Western blot using anti-CRD Ab (Magez et al., 2001).

2.5. Human sera and purified antibodies

2.5.1. Human sera

We used sera from patients with HAT or uninfected subjects (endemic area controls) living in Boko Songho (Bouenza focus of Congo). Clinical examination, parasite detection, and serological analyses were previously described (Okomo-Assoumou et al., 1995a).

2.5.2. Affinity-purified antibodies

Beads of CNBR activated Sepharose 4 fast flow, (Amersham Pharmacia Biotech AB, Uppsala, Sweden) were used according to the manufacturer instructions. WE was synthesized by linking L-tryptophan (W) to BSA via glutaraldehyde (G) as previously described (Geffard et al., 1985a; Okomo-Assoumou et al., 1995a). WE and VSG (from LiTat 1.3) were coupled to beads and washed in carbonate buffer. Five milliliter of human sera were incubated with beads overnight at 4 °C. Unabsorbed fractions were collected and analysed by ELISA or Western blot. VSG-bound Ig or WE-bound Ig were eluted with 0.1 M glycine, pH 2.5, and immediately neutralized with 0.01 M Tris, pH 8.8, and secondly with 0.1 M triethylamine, pH 11.5. After dialysed overnight against PBS, pH 7.5, at 4 °C and 50% $(NH_4)_2SO_4$ precipitation as previously described (Chagnaud et al., 1992; Loirand et al., 1992), protein concentrations were determined by the Bradford method (Bradford, 1976). For comparative studies, sera, run-through fractions, bound, and eluted fractions, were brought to the same volume.

2.6. Rabbit immunoglobulins

Rabbits were immunized subcutaneously with 500 μl of soluble antigen (1 mg/ml) from *T. b. gambiense* "Féo"(ITMAP/1893). Immunoglobulins (Ig) from preimmune and immune sera were purified by ammonium sulphate precipitation (Mons and Geffard, 1987).

Affinity-purified immunoglobulins from sera of rabbits immunized with BSA-conjugated catecholamines [dopamine, (Mons and Geffard, 1987)], indoleamines [tryptophan (W), hydroxytryptophan (HW), methoxytryptophan, and serotonin, (Geffard et al., 1984, 1985a)], were also used.

2.7. Enzyme linked immunosorbent assay (ELISA)

Anti-WE Ab in human sera were detected by an ELISA technique using antigens from bloodstream forms of *T. b. gambiense* ("Féo" and "LiTat 1.3") (Okomo-Assoumou et al., 1995a). Antigens were coated at 2 μg/ml. Human sera were diluted at 1/4000 and goat anti-human IgM labelled with horseradish peroxidase (Institut Pasteur Production, Paris, France) was diluted at 1/5000. After the addition of *o*-phenylenediamine and H_2O_2, the reaction was stopped by H_2SO_4, and the absorbances were measured at 490 nm with a Multiskan spectrophotometer (Dynatech, VA, USA).

2.8. Polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting procedures

Reducing SDS–PAGE was performed using 8–15% gradient polyacrylamide slab gels (Laemmli, 1970; Merril et al., 1984). Proteins were incubated in a sample buffer containing 30% glycerol, 4% SDS and 3% β-mercaptoethanol (β-ME) for 5 min at 100 °C. Electrophoresis was carried out at 24 mA for 3–4 h. Proteins were stained with Coomassie blue and apparent molecular weights were obtained by comparison with standard protein markers (Electrophoresis Calibration Kit, Pharmacia, France).

Transfers were performed after SDS–PAGE. Materials were transferred on PVDF membranes (0.45 μm pore size, Immobile-P, Millipore) as described by Towbin et al. (1979). After blocking of the non-specific binding and washings with TBS containing 0.05% Tween 20 (TBS–Tween), the membranes were immersed for 16 h at 4 °C under agitation with rabbit or human Ab, diluted 1:4000. After 3 washings, the membrane were immersed for 2 h at 37 °C with stirring in biotinylated swine Ig to rabbit or human (DAKO, Denmark) diluted 1:1000. After 3 washings, the membranes were immersed for 2 h at room temperature in peroxidase-conjugated streptavidin (Dako) diluted at 1:10,000, and peroxidase activity was visualized by adding diaminobenzidine and H_2O_2 (Chagnaud et al., 1992).

2.9. Statistical methods

Statistical significance was evaluated using the nonparametric Mann–Whitney *U* test. Correlation between groups was made using the Spearmann correlation nonparametric test. Any *P* value lower than 0.05 was considered to be significant.

3. Results

3.1. Specific recognition of parasite antigen by anti-WE antibodies

Affinity-purified rabbit Ab directed to a chemically defined WE were used. The high avidity and specificity of these Ab were confirmed as previously described (Geffard et al., 1985b; Geffard et al., 1987). Western blotting using whole bloodstream trypanosome antigen and affinity-purified rabbit anti-WE Ab revealed an intense recognition of the major trypanosome antigen. Similar results were

obtained with soluble antigens. The immunoreactivity of a panel of affinity-purified rabbit Ab directed against conjugated antigens both related and unrelated to WE was assayed on Ag from *T. b. gambiense* "Féo" after SDS–PAGE separation. Rabbit Ab to soluble Ag from *T. b. gambiense* "Féo" and anti-WE Ab were used as controls. No binding was found with Ab directed against an Ag related (conjugated 5-hydroxytryptophan) and one unrelated (conjugated Dopamine) to WE, (Fig. 1A). No binding was also found with affinity-purified rabbit Ab directed against conjugated serotonin or conjugated methoxytryptophan and with Ig from normal rabbits (data not shown).

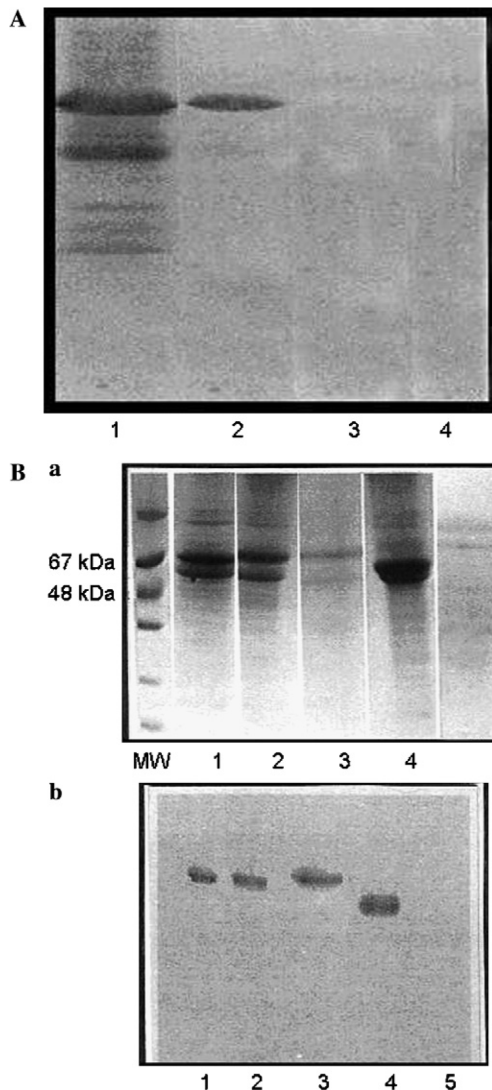


Fig. 1. Comparison of the reactivity of rabbit Ab directed to trypanosome antigens and chemically defined epitopes on parasite antigens by Western blotting: (A) The blots were probed with rabbit antibodies directed against: lane 1, soluble antigens from "Féo"; lane 2, WE; lane 3, 5-hydroxytryptophan; lane 4, dopamine. (B) Identification of trypanosome antigens and tryptophan like (WE) epitope: (a) Coomassie blue staining of SDS–PAGE from *T. b. gambiense* (lane 1, "Féo"; lane 2, "OK"; lane 3, "Biyamina"), *T. b. brucei* (lane 4, AnTat 1.1), and *T. cruzi* (5) soluble antigens. (b) Western blotting with affinity-purified rabbit anti-WE Ab. MW, molecular weight markers (94, 67, 43, 30, 20.1, and 14.4 kDa).

3.2. Identification of parasite antigen(s) recognized by anti-WE antibodies

Soluble parasite antigens from bloodstream *T. b. gambiense* ("Féo", "OK" and "Biyamina"), *T. b. brucei* (AnTat 1.1), and *T. cruzi* (TC SO4) were fractionated by SDS–PAGE and stained by Coomassie blue (Fig. 1B(a)). These antigens were transferred on to Immobilon membrane and tested with rabbit anti-WE Ab. An Ag bearing a WE was identified in various strains of the *brucei* group, whereas no Ag from *T. cruzi* was recognized (Fig. 1B(b)). Similar results were obtained by using whole trypanosome antigens from the *brucei* group whereas no Ag from *T. musculi* was detected (data not shown).The apparent molecular weights of the Ag (60 kDa) recognized by anti-WE Ab match those of the major bloodstream parasite Ag (VSG).

3.3. Absence of recognition of procyclic antigens by anti-WE antibodies

The presence of WE was investigated on procyclic antigens from *T. b. gambiense* to *T. b. brucei*. Ag from *T. b. gambiense* (Mabaya, Murraz 15, Dal 972 and NGB) to *T. b. brucei* (MPAM/TZ/71/C126 and MOVS/ZR/86/SH86) were fractionated by SDS–PAGE and transferred into Immobilon membrane and tested with rabbit anti-WE Ab. No Ag from procyclic forms of trypanosomes was recognized by these Ab whereas strong signals were obtained with Ag from trypomastigote forms ("Féo" and "Biyamina") used as controls (data not shown).

3.4. Tryptophan-like epitope is borne by VSG of different trypanosomes species

VSG purified from different bloodstream trypanosomes showed a slight difference in their molecular weight, after SDS–PAGE (Fig. 2A). Analysis of anti-WE Ab immunoreactivity on these purified VSG clearly showed that VSG is recognized and that it corresponds to parasite Ag bearing a WE in comparison with the band identified in whole Ag extract (lane1) (Fig. 2B). The use of purified VSG confirmed that VSG is the parasite Ag bearing WE which is found in several VSG from *T. b. gambiense*, *T. b. brucei*, and *T. evansi* (Fig. 2B, lanes 2–7).

Trypanosoma brucei brucei AnTat 1.1 derived soluble VSG with GPI anchor and VSG lacking the GPI anchor were checked for their reactivity with affinity-purified rabbit anti-WE Ab. Anti-WE recognition was not abrogated by the removal of the GPI anchor (data not shown).

3.5. Cross reactivity of WE

Purified Ab from patient sera were eluted from WE-bound column. They gave strong immunoreactive signals on both VSG (LiTat 1.3) and WE by Western blotting (Fig. 3A). Purified anti-VSG Ab eluted from VSG bound

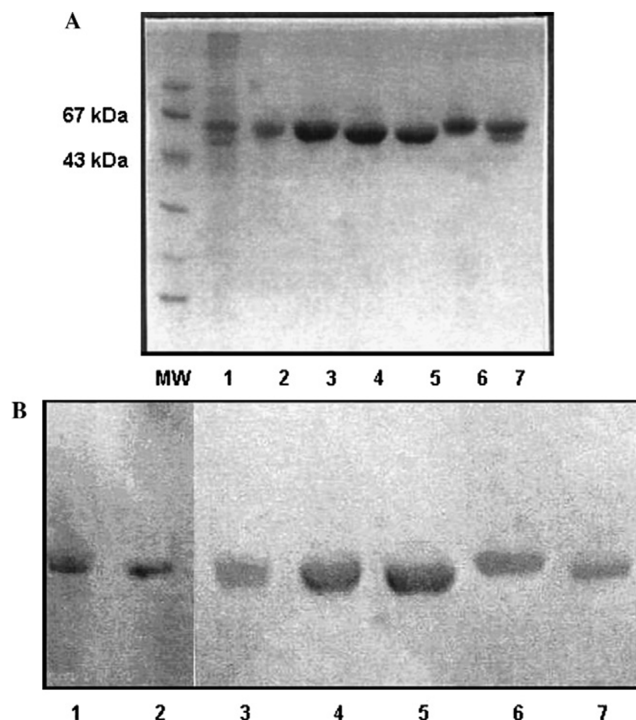
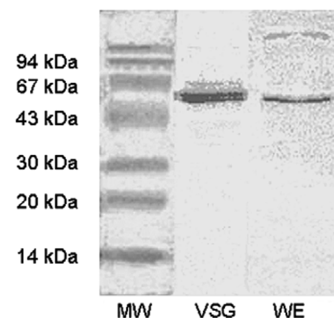


Fig. 2. Analysis of anti-WE Ab binding on purified VSGs : (A) Coomassie blue staining of various VSGs (B). Western blotting with affinity-purified rabbit anti-WE: lane 1, soluble antigens from "Féo"; lane 2, purified VSG from *T. b. gambiense* ("Féo"); lane 3, LiTat 1–3; lane 4, LiTat 1–5; lane 5, LiTat 1–6; lane 6, *T. b. brucei*; and lane 7, *T. evansi*. MW, molecular weight markers (94, 67, 43, 30, 20.1, and 14.4 kDa).

A purified anti-WE Ab assessed on VSG and WE



B purified anti-VSG Ab assessed on VSG and WE

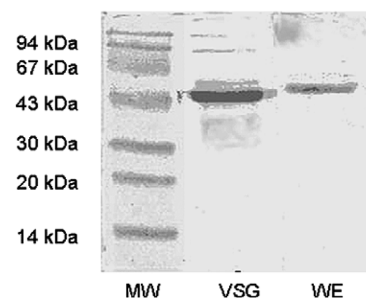


Fig. 3. Western blotting with affinity-purified anti-WE and anti-VSG Ab from HAT sera. (A) Binding of purified anti-WE on VSG (LiTat 1.3) and WE. (B) Binding of purified anti-VSG on VSG (LiTat 1.3) and WE.

column gave immunoreactive signals on both VSG and WE (Fig. 3B).

The whole HAT sera were first exhausted on VSG and on WE and the remaining immunoreactivity on each epitope was assayed by ELISA (Fig. 4). After absorption on VSG, no remaining immunoreactivity was found on both VSG on WE. After absorption on WE, no immunological signals were detected using WE whereas a significant immunoreactivity was detected on VSG. In the same conditions, no immunological signals were obtained by using sera from control subjects, whereas strong positivities were obtained with purified antibodies.

4. Discussion

High levels of Ab directed to a chemically defined epitope (L-tryptophan linked to BSA with glutaraldehyde, WE) were found in HAT sera but were absent in sera of patients with malaria, HIV infection, Chagas or Parkinson disease (Okomo-Assoumou et al., 1995a). The presence of a WE borne by trypanosomes of the *brucei* group was identified by using affinity-purified rabbit anti-WE Ab. A specific recognition of the major Ag (VSG) of the bloodstream forms of trypanosomes of the *brucei* group was observed using these Ab, whereas no recognition was observed using rabbit Ab directed to Ag both related and unrelated to WE. This Ag was not detected in procyclic forms of *T. b. gambiense* and *T. b. brucei*, which are devoid of VSG, and in stercorarian trypanosomes (*T. cruzi* and *T. musculi*).

The immunoreactivity of rabbit anti-WE Ab was investigated on protein extracts from various strains, and belonging to different taxa of trypanosomes. These Ab reacted with a major Ag present in all Ag preparations from the *brucei* complex, whereas no Ag from *T. musculi* or from *T. cruzi* was recognized. The recognized Ag had a molecular weight of about 60–65 kDa and, using purified VSG, was identified as the VSG molecule. A cross reactivity of WE and VSG was demonstrated by using affinity-purified anti-WE and anti-VSG Ab from HAT patients. Immune reactivity of anti-WE-depleted HAT sera showed that WE was one epitope borne by VSG. This epitope is conserved in VSG lacking GPI anchor and not recognized by anti-CRD.

African trypanosomes are entirely covered by VSG, which constitutes at least 95% of its surface proteins, and as much as 10% of its cell proteins (Cross, 1984). Despite the variability in the sequence of these molecules, a common WE is found in VSG of the *brucei* complex. Sequence amino acid analysis showed that the N-terminal domain is extremely diverse except for the relative locations of L-cysteine residues which are conserved (Cross, 1984). The C-terminal domain, however, exhibits a degree of sequence conservation (Holder and Cross, 1981; Matthyssens et al., 1981). VSG of *T. brucei* has been classified in four classes based on peptide homology in C-terminal domains. L-W is mainly found in conserved aromatic amino acid of

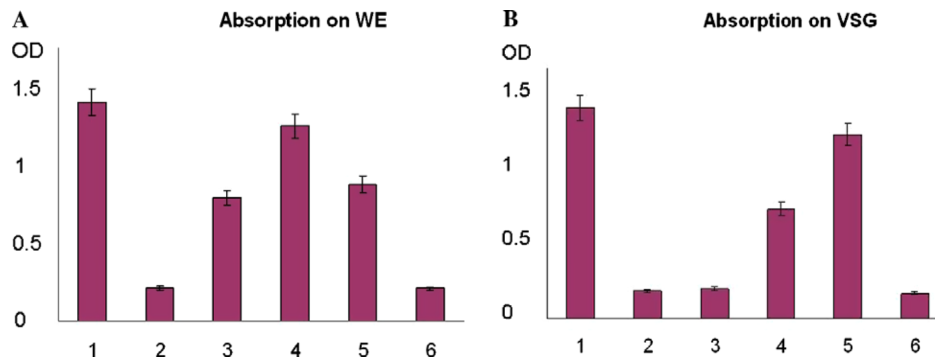


Fig. 4. Immunoenzymatic assays of residual antibody from HAT sera after absorption on (A) WE and (B) VSG. 1, Non-absorbed HAT sera; 2, run-through fraction assayed on WE; 3, run-through fraction assayed on VSG; 4, bound and eluted fraction on WE; 5, bound and eluted fraction on VSG; 6, control sera from non-HAT patients.

the C-terminal domain of VSG from *T. brucei* (Carrington et al., 1991). It is often located in the flanking regions of positionally conserved cystein. In their hydrophobic regions, a tryptophan residue is one of the conserved flanking residues of cyteine residues in the C terminal domains of VSG. The solution structure of VSG type 2 C-terminal domain from MITat1.2 has been recently reported. This domain is comprised of a compact core flanked by two unstructured regions. Disulfide bonds are flanked by 2 hydrophobic residues (tryptophan and leucin) (Chattopadhyay et al., 2005). These residues are conserved in various VSG type 2 C-terminal domains. A tryptophan residue is conserved in *Trypanosoma congolense* VSG (Strickler et al., 1987; Rausch et al., 1994). X-ray data of two VSG, which have different amino acid sequences, revealed a surprising similarity in the tertiary structure of their N-terminal domain (Down et al., 1991; Blum et al., 1993). This suggests that common structural motifs may occur in all VSG, especially in those of the *T. b. brucei* type. Comparisons of DNA sequences of VSG genes from *T. b. gambiense* and other *T. brucei* sp. have shown a high level of homology, suggesting recent gene flow (Bromidge et al., 1993).

A subpopulation of B cells, identified by the expression of high levels of surface immunoglobulins and of CD5 in humans and Ly-1 in mice, is responsible for most serum IgM (Kipps, 1990). These CD5 B cells produce autoantibodies, and antibodies to thymus-independent antigens. The presence of low titers of anti-VSG Ab in the sera of non-infected humans and animals might be linked to these cells (Müller et al., 1996). In *T. congolense*-infected cattle, a dramatic increase in these cells was measured and correlated with increase in serum immunoglobulins and in absolute number of B cells (Naessens and Williams, 1992). An induction of these CD5 B cells by VSG-borne epitopes might account for the production of anti-WE Ab of the M isotype in HAT. These Ab may be directed against parasite antigens or autoantigens or may be natural antibodies amplified by the trypanosome infection (Buza and Naessens, 1999).

A lot of autoantibodies are detected in HAT. They might participate in pathophysiological mechanisms in HAT. Anti-galactocerebroside Ab, which are also found in HAT, mediate the demyelination of the CNS *in vivo* (Ozawa et al., 1989). Antibodies directed against a WE epitope of trypanosomes might cross react with other antigens, including host antigens. Ab directed against the *CNS* can be synthesized *in situ* or go into the central nervous system through alterations of the blood brain barrier, which has a higher permeability in HAT (Philipp et al., 1994). They may act in synergy with other immune elements, such as TNF-α, whose level is persistently increased in HAT (Pentreath, 1989; Okomo-Assoumou et al., 1995b). In CSF, some chemokine levels were elevated in the latter stages of HAT (Courtioux et al., 2006). Therefore, recruited cells in brain might also be involved and participate to HAT pathophysiology. A retrograde degeneration of serotoninergic neurons has been noted in *T. b. brucei*-infected mice (Ormerod and Hussein, 1987; Ormerod et al., 1988). Anti-WE Ab might participate to the alterations of the serotoninergic pathways, especially those involved in somnogenesis.

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