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HUMAN/VECTOR RELATIONSHIPS DURING HUMAN AFRICAN TRYPANOSOMIASIS: INITIAL SCREENING OF IMMUNOGENIC SALIVARY PROTEINS OF GLOSSINA SPECIES

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Abstract. The morbidity and mortality of vector-borne diseases is closely linked to exposure of the human host to vectors. Qualitative and quantitative evaluation of individual exposure to arthropod bites by investigation of the specific immune response to vector saliva would make it possible to monitor individuals at risk of vectorial transmission of pathogens. The objective of this study was to evaluate and compare the antibody (IgG) response to saliva from uninfected Glossina species, vectors, or non-vectors of Trypanosoma brucei gambiense by detecting immunogenic proteins in humans residing in an area endemic for human African trypanosomiasis in the Democratic Republic of Congo. Our results suggest that the immunogenic profiles observed seemed specific to the Glossina species (vector or non-vector species) and to the infectious status of exposed individuals (infected or not infected). This preliminary work tends to support the feasibility of development of an epidemiologic tool based on this antibody response to salivary proteins.

INTRODUCTION

The chronic form of sleeping sickness (human African trypanosomiasis [HAT]) caused by Trypanosoma brucei gambiense is found in western and central Africa and is transmitted to humans by bites of the Glossina vector (tsetse fly). Among vector Glossina species, Glossina palpalis, G. tachinoides, and G. fuscipes are able to transmit T. b. gambiense. This slowly progressing fatal disease often remains undiagnosed because of lack of specific symptoms observed during its initial stage, and the infection is readily confused with other febrile illnesses, especially malaria. The World Health Organization (WHO) has recommended systematic screening of populations by serologic survey to diagnose infected individuals and to control HAT transmission. In the mid-1980s, the incidence of HAT increased to alarming levels comparable to those of the early 20th century, particularly in the Democratic Republic of Congo, and it now represents a major public health problem. This re-emergence can be partly explained by laxity in control and research efforts. In view of this alarming situation, intensification of active case detection and treatment was initiated by the national control programs of disease-endemic countries and results seem promising; WHO recently reported a decrease in the number of new reported cases.

The morbidity and mortality of sleeping sickness is closely linked to transmission of the pathogen by the vector, and is therefore associated with exposure of the human host to infected Glossina bites. However, the relationship between the presence of vectors and the prevalence of the disease is complex, and other parameters must be considered when evaluating risk of infection. This complexity underlines the importance of developing new tools to identify populations exposed to a high risk of transmission and which could enable optimization of epidemiologic surveillance and identification of individuals who may benefit from monitoring. Currently, exposure of populations to the Glossina vector is evaluated by entomologic methods (e.g., capture by traps), but such methods cannot evaluate heterogeneous individual exposure.

During blood feeding, insects inject salivary proteins (sialome), primarily to counteract the host hemostasis response induced by the bite. The pharmacologic properties of these proteins, such as platelet aggregation inhibitors, vasodilators, and inhibitors of blood coagulation, are necessary for blood feeding. Some of these salivary proteins also have immunogenic characteristics (immuno-sialome) that enable initiation of a specific immune response. Based on these immune properties, studies have shown the potential of markers of exposure to vector-borne diseases by evaluation of a specific antibody response to salivary proteins in individuals exposed to the arthropod vector. The first example of an epidemiologic indicator was reported in the United States in individuals infected with Borrelia burgdorferi, the causative agent of Lyme disease. The level of IgG antibody specific for Ixodes dammini tick saliva was higher in individuals at high risk of developing Lyme disease. Moreover, specific IgG1 and IgG4 isotypes of antibody to Triatoma salivary antigens have been detected in individuals exposed to Chagas disease and the level of specific IgG4 of Aedes aegypti saliva might be a marker of intense exposure to Aedes bites. It has been also suggested that immune response to sand fly recombinant salivary gland protein may be a good marker of vector exposure. Recently, it has been shown that children from a malaria-endemic area in Senegal developed a specific IgG response to Anopheles gambiae saliva that was positively associated with the degree of exposure to vector bites evaluated by classic entomologic studies. In addition, high levels of IgG antibody to saliva appeared to be a predictive indicator of malaria morbidity.

Few studies have explored the immunogenic properties of Glossina saliva in sleeping sickness. Immediate or delayed
hypersensitivity responses have been observed in humans and rabbits exposed to tsetse fly bites, suggesting immunogenic properties of *Glossina* saliva. Another study attempted to identify an epidemiologic indicator of human contact with *Glossina* by detection of specific antibody of *Glossina* salivary glands in human sera, but the immunologic techniques used (immunoelectrodiffusion) failed to detect an immunologic response.

The present study represents a new attempt to evaluate the immune response to tsetse fly bites in human populations in an area endemic for sleeping sickness. The objective of the study was to detect immunogenic salivary proteins of *Glossina* (vector and non-vector species) and to compare the profiles of these immunogenic proteins, taking into account the infectious status of individuals living in an area endemic for HAT.

**MATERIALS AND METHODS**

**Studied population.** The study was conducted using sera from individuals in the HAT-endemic area of Bandundu in the Democratic Republic of Congo. The status of 74 individuals included in the study was defined using serologic (card agglutination trypanosoma tests for *T. b. gambiense* and trypanolysis test), parasitologic, and molecular (polymerase chain reaction) investigations. Stage diagnosis was carried out for all sleeping sickness cases (n = 19), by counting white blood cells present in the cerebrospinal fluid (CSF). These results led to defining three groups of individuals: 1) the ENI group, which included exposed but non-infected individuals (n = 55) who had no positive test results; 2) the P1 group, which included exposed and infected patients in the first stage of the disease (n = 7) who had positive test results and < 5 white blood cells/μL in CSF; and 3) the P2 group, which included exposed and infected patients in the second stage of the disease (n = 12) who had positive test results and ≥ 5 white blood cells/μL in the CSF. A negative control (CHU) group included individuals (n = 16) who were never exposed to *Glossina* spp. bites; it included personnel from Lapeyronie Hospital (Montpellier, France). The study adhered to the ethical principles defined by the Helsinki Declaration, and was reviewed and approved by local traditional authorities and by the local Ethical Committee of the Democratic Republic of Congo (Public Health Ministry, 2001). All individuals enrolled in this study signed an informed consent form.

**Saliva collection.** Saliva samples from uninfected male and female *Glossina* bred in an insectarium (Unité de Recherche 177, Institut de Recherche pour le Développement) were collected. The tsetse flies were enclosed in a tube by a mosquito net and the tube was placed above a drop of salivation buffer (10 mM HEPES, 150 mM NaCl, and 5 mM EDTA, pH 7.2) on warm slides (37°C). After 10 minutes of salivation, the saliva solution was collected, pooled, and stored at −20°C before use. The saliva of four tsetse flies species was collected: three vector species of *T. b. gambiense* (G. fuscipes fuscipes, G. tachinoides, and G. palpalis gambiensis) and one non-vector species of *T. b. gambiense* (G. morsitans morsitans).

**Silver staining.** Silver staining was achieved, after which development was stopped with 5% acetic acid.

**Immunoblotting.** The proteins separated by one-dimensional electrophoresis were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories) in Tris-glycine buffer at 100 mA for one hour and at 300 mA for one hour. The membrane was then washed three times with Tris-buffered saline (TBS) and blocked with 5% skim milk in TBS for one hour. The membrane was washed three times with TBS containing 0.1% Tween 20 and three times with TBS, and then equilibrated with 2% skim milk in TBS for 20 minutes. Immunogenic proteins were detected by incubation of the membrane with human sera (1:150) overnight at 4°C. Mouse anti-human IgG conjugated to alkaline phosphatase (Sigma-Aldrich) were added at a dilution of 1:5,000. Before and after addition of the secondary antibody, the membrane was washed three times in TBS containing 0.1% Tween 20 and three times with TBS. The membrane was incubated with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate (Sigma-Aldrich) until a desired level of staining was achieved and digitized by a personal densitometer SI (Amer sham Biosciences, Piscataway, NJ).

**RESULTS**

**Glossina salivales.** The profiles of salivary proteins (salivales) from male and female *Glossina*, both vector or non-vector species, are shown in Figure 1. Approximately 15–20 bands with molecular weights of 11–240 kD were observed in saliva samples of each *Glossina* species. Saliva of the three potential vector species of *T. b. gambiense* (G. fuscipes fuscipes, G. tachinoides, and G. palpalis gambiensis) had many common bands (approximately 24, 27, 33, 37, 39, 54, 60, 70, 100, 110, 130, and 150 kD) with some variations in intensity of silver staining. Some of these proteins were also detected in *G. morsitans morsitans* saliva, but non-vector saliva contained more proteins with molecular weights of approximately 50–72
because this species is the main vector of saliva (50, 55, 65, and 72 kD; Figure 2). We evaluated the immunogenic profile of salivary proteins in male. The composition of species. Shown are three vector species (Glossina palpalis gambiensi [G.pg], G. fuscipes fuscipes [G.ff], G. morsitans morbitans [G.mm]) and 1 non-vector species (G. morsitans morbitans [G.mm]) of Trypanosoma brucei gambiense. Saliva were analyzed by 4–15% gradient one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions and silver staining. Molecular mass markers (MW) are shown on the left in kilodaltons (kDa).

We evaluated the immunogenicity of salivary proteins saliva. The protein profile of Glossina saliva appeared to be species specific, with strong similarities between vector species and important differences in non-vector species G. morsitans morsitans. The composition of saliva appeared similar in females and males for all Glossina species.

Detection of immunogenic proteins according to Glossina species. We evaluated the immunogenic profile of Glossina salivary proteins (immuno-sialome) by immunoblotting with a pool of sera of exposed but non-infected persons (ENI group) (Figure 2). An IgG antibody response specific to saliva samples of each Glossina species was detected and the immunogenic profile appeared to be different according to the Glossina species.

For each saliva sample, 10–15 bands were detected. Some bands (37, 54, 60, 70, 95, and > 170 kD) were common to the four species tested. However, the immunogenic profile of non-vector saliva was different from that obtained with saliva of vector species. This difference was closely linked to the immunogenicity of specific proteins observed only in G. morsitans morsitans saliva (50, 55, 65, and 72 kD; Figure 2). We also detected a specific band of approximately 42 kD in G. fuscipes fuscipes saliva (Figure 2). In addition, the intensity of immunogenic bands differed according to Glossina species. For example, the double band of approximately 130 kD detected in the saliva of the four species (Figure 1) showed higher immunogenic characteristics only in G. morsitans morsitans. In addition, we observed a signal that had a staircase appearance at the low molecular weight range in the vector species. No relevant differences were observed in the immunogenic profile between male and female saliva for any of the Glossina species. Thus, immunogenicity of salivary proteins did not appear to be sex dependent for any Glossina species.

Detection of immunogenic proteins of G. fuscipes fuscipes saliva according to the trypanic status of exposed individuals. We evaluated the immunogenicity of salivary proteins during chronic evolution of sleeping sickness by comparing the immune profiles of G. fuscipes fuscipes saliva between individual groups (pools of sera) with varying trypanic status: exposed but non-infected persons (ENI group), infected individuals (P1 and P2 groups), and negative controls (CHU group). Results were obtained for saliva from G. fuscipes fuscipes because this species is the main vector of T. b. gambiense in the Democratic Republic of Congo. Figure 3 shows results for male G. fuscipes fuscipes saliva; similar results for each group were observed in females and mixed (male plus female) saliva samples. We observed four major bands (37, 54, 60, and 150 kD) in the three exposed groups regardless of the HAT status. In addition, four minor bands with low immunogenicity (90, 95, 110, and 125 kD) were detected in these three groups. The infected groups (P1 and P2) showed similar immunogenic profiles. In contrast, a difference in the immunogenic profiles was detected between infected patients (P1 and P2 groups) and exposed but non-infected individuals (ENI group). Some salivary proteins (four bands between 37 kD and 42 kD) were immunogenic only in the ENI group. Surprisingly, weak intensity of three immunogenic proteins (54, 60, and 150 kD), the major 37-kD band, and the previously observed staircase signal were observed in the CHU group.

Analysis of the individual immunogenic profile in G. fuscipes fuscipes saliva. We investigated the immunogenic profile of exposed but non-infected (ENI) and infected (P1) individuals to mixed G. fuscipes fuscipes saliva (female plus male). This study was carried out on nine ENI patients (Figure 4A) who were randomly selected, and on all persons in the P1 group (n = 7; Figure 4B).

The profiles of individuals in the ENI group were heterogeneous, which suggested variability in the immunogenicity of salivary proteins among individuals with the same trypanic status. The four major immunogenic bands (37, 54, 60, and...
and two minor bands with low immunogenicity (90 and 110 kD) differed in intensity among persons in the ENI group. In addition, such individual variations were also observed for ENI-specific proteins of approximately 37–42 kD.

In contrast, a similar profile of immunogenic salivary proteins was observed among persons in the P1 group. Most individuals in this group showed a specific IgG antibody response to the four major bands (37, 54, 60, and 150 kD) with particularly high immunogenicity for three of them (37, 60, and 150 kD). In addition, this homogeneity of the profile of P1 individuals was observed with two bands having low immunogenicity (70 and 110 kD). Most individual profiles (ENI and P1) had the previously observed staircase signal at low molecular weights.

**DISCUSSION**

In the present study, specific immune responses to salivary proteins of *Glossina* were detected in humans residing in an area endemic for a sleeping sickness. We demonstrated that the specific antibody response to these immunogenic proteins was dependent on the *Glossina* species and the infection status of studied individuals.

Previous studies on *Glossina* saliva had been carried out principally on salivary glands (cDNA bank or protein extraction) of non-vector *Glossina* species of *T. b. gambiense* (*G. morsitans morsitans* and *G. morsitans centralis*), but salivary glands contain structural proteins that are not secreted during biting. Nevertheless, they enable identification of some proteins with pharmacologic properties involved in inhibition of the human host hemostasis response. In the present study, the salivation technique used enabled analysis of biologic material similar to saliva injected in the vertebrate host during natural blood feeding. This enabled identification of components involved in the host/vector relationship. The sequencing of an extensive set of expressed sequence tags of the *G. morsitans morsitans* salivary gland is currently being carried out and should confirm the effectiveness of our salivation technique.

In the present study, analysis of sialomes identified 15–20 bands by one-dimensional SDS-PAGE. It would be useful to
determine whether one band corresponds to one or more proteins. Such identification could be performed by separating the salivary proteins by two-dimensional electrophoresis and analyzing them by mass spectrometry. However, we have shown that numerous bands are common to Glossina species, but other bands are present only in potential vector species (G. fuscipes fuscipes, G. tachinoides, and G. palpalis gambiensis) and non-vector species (G. morsitans morsitans) of T. b. gambiense. This difference in saliva composition, including the presence or absence of certain salivary proteins, could be involved in vector capacity of the Glossina species. For example, a salivary protein may be essential for continuation of the parasite maturation cycle in the vector, as suggested in the Anopheles mosquito.28

To our knowledge, the present study is also the first description of Glossina immuno-sialome carried out by investigating the specific human IgG response to Glossina saliva in a population exposed to HAT. Previously, hypersensitivity reactions of an immediate or delayed type were observed in humans, but specific salivary components involved had not been identified.18,20 Moreover, a previous study described sialome and immunogenic salivary proteins of G. morsitans centralis that were detected by IgG antibodies from sensitized rabbits.29

Comparison of immuno-sialomes of the four Glossina species shows a difference in the profile of immunogenic proteins according to species. This difference was greater between Glossina vector species or non-vector species of T. b. gambiense, as we observed in sialome analyses. Most of these immunogenic proteins were common to the four Glossina species. Nevertheless, the immunoblotting approach distinguished immunogenic proteins specific to Glossina species (42 kD specific for G. fuscipes fuscipes and 50, 55, 65, and 72 kD specific for G. morsitans morsitans). Species-specific antigens were also identified in Phlebotomus and Aedes species.29,30

The difference in immunogenic protein composition in Glossina species may also be involved in transmission of the parasite. For example, the immune response induced by specific proteins of non-vector G. morsitans morsitans (50, 55, 65, and 72 kD) could play a role in preventing infection permissiveness. It has been demonstrated that the specific immune response to one Phlebotomus salivary antigen (PpSP15) can confer protection against the development of leishmaniasis in animal models.31

Another difference between vector and non-vector Glossina species was also observed. A signal (<37 kD) in the form of bands in a staircase pattern was only detected in saliva of Glossina vector species (G. fuscipes fuscipes, G. tachinoides, and G. palpalis gambiensis). One hypothesis is that this signal could be an artifact of handling. In addition, endosymbiont Sodalis glossinidus bacteria can be found at the level of salivary glands of certain Glossina species, but are absent in G. morsitans morsitans salivary glands.32 Another hypothesis is that proteins of these bacteria are secreted during Glossina bites and induce a specific immune response in humans. The difference in immunogenic protein composition in proteins (sialome) and immunogenicity were commonly detected.

Since the mid 1990s, several studies have investigated the immune response of mammalian hosts to arthropod bites.35 Qualitative and quantitative evaluation of specific human antibody responses to arthropod salivary proteins may be a useful marker of exposure to vector-borne diseases, similar to markers for malaria in Senegal and for Chagas disease and visceral leishmaniasis in Brazil.12,16,17

Selection of immunogenic proteins for elaboration of an immune marker of exposure to Glossina requires knowledge of vector saliva composition in proteins (sialome) and immunogenic characteristics of these proteins (immuno-sialome). Candidate proteins must be expressed in saliva of most Glossina species, or at least be common to vector Glossina species.
In several hematophagous species, the male does not take a blood meal, which is reflected in salivary morphology and secretions. Both female and male Glossina could be vectors of Trypanosoma, and we have shown that both sexes have similar immunogenic profiles of salivary proteins. Since the observed immune response must be specific to Glossina salivary antigens, the protein containing potential common epitopes with other vector arthropods would have to be eliminated from the pool of candidate proteins. Furthermore, immunogenic proteins specific to a Glossina species (42 kD for G. fuscipes fuscipes and 50, 55, 65, and 72 kD for G. morsitans morsitans in our study) should also be markers of distinguishing specific exposure to the bite of one Glossina species.

Candidate proteins should have immunogenic properties enabling detection of specific antibodies in all persons exposed to Glossina bites, whether or not the persons are infected. Several immunogenic proteins of interest were emphasized in our results. Major proteins were identified in ENI and PI individuals, including 37-, 60-, and 150-kD bands. However, these bands, except for the 37-kD band, also appeared to be immunogenic in the unexposed CHU group but their immunogenicity was low. Among the minor proteins, only the 110-kD protein was immunogenic in most individuals exposed to Glossina bites, but it showed weak immunogenicity in our study. We have not identified a marker protein, and our results represent a first attempt to characterize the immunogenic profile of Glossina salivary proteins in an exposed population. Nevertheless, this study indicates the feasibility of elaboration of an immuno-epidemiologic marker of exposure based on antibody response to salivary proteins.

Further studies on other sleeping sickness transmission foci are needed to confirm these results. The immuno-proteomic approach using two-dimensional electrophoresis could identify immunogenic proteins specific for exposure to Glossina bites and could be used for mass spectrometric characterization of proteins. After immunogenic proteins are identified, selected proteins could be produced in their recombinant form and a quantitative method (enzyme-linked immunosorbent assay) could be used to evaluate specific individual antibody titers to these proteins in the framework of large-scale immuno-epidemiologic studies. These immunologic results could be compared with exposure (as evaluated by an entomologic approach) and morbidity data.

The development of an immuno-epidemiologic marker of exposure to Glossina bites could represent a tool complementary to those currently available, which include diagnostic tests enabling evaluation of morbidity and environmental data (satellite data, climate models, entomologic information, and cartography) that define where and when populations have been exposed to vectors and parasites. Such a marker could be used to estimate the exposure of individuals to Glossina bites and be an indicator of risks of HAT transmission.

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