Tsetse Fly Saliva Accelerates the Onset of *Trypanosoma brucei* Infection in a Mouse Model Associated with a Reduced Host Inflammatory Response

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Tsetse flies (*Glossina* sp.) are the vectors that transmit African trypanosomes, protozoan parasites that cause human sleeping sickness and veterinary infections in the African continent. These blood-feeding dipteran insects deposit saliva at the feeding site that enables the blood-feeding process. Here we demonstrate that tsetse fly saliva also accelerates the onset of a *Trypanosoma brucei* infection. This effect was associated with a reduced inflammatory reaction at the site of infection initiation (reflected by a decrease of interleukin-6 [IL-6] and IL-12 mRNA) as well as lower serum concentrations of the trypanocidal cytokine tumor necrosis factor. Variant-specific surface glycoprotein-specific antibody isotypes immunoglobulin M (IgM) and IgG2a, implicated in trypanosome clearance, were not suppressed. We propose that tsetse fly saliva accelerates the onset of trypanosome infection by inhibiting local and systemic inflammatory responses involved in parasite control.

African trypanosomes are extracellular protozoan flagellates that infect a broad range of vertebrate hosts, including humans, and rely for their transmission on tsetse flies (*Glossina* sp.), which are obligate blood-feeding organisms (62). In the mammalian host, the parasites establish controlled growth to ensure survival and optimal transmission. In order to escape the adaptive immune system, trypanosomes undergo antigenic variation by altering their major surface antigen, the variant-specific surface glycoprotein (VSG) (6). Beside this immune evasion mechanism, trypanosomes have been shown to modulate immune functions of macrophages (8, 43), T lymphocytes (56), and B lymphocytes (2). The main host immune effectors involved in parasite control are considered to be trypanosome-specific antibodies (30) and the cytokine tumor necrosis factor (TNF) (33). Concerning trypanosome-specific antibodies, increased VSG-reactive immunoglobulin M (IgM) and IgG2a antibody isotypes have been associated with improved control of trypanosome infections (34, 55). TNF, as a host cytokine, was released from activated macrophages in response to stimulation by soluble VSG (sVSG) and membrane-bound VSG (11, 35). TNF was demonstrated to have trypanocidal properties for certain trypanosome stocks and to be associated with the occurrence of immune pathology in infected animals (33). As such, direct or indirect modulation of parasite-specific antibody induction and TNF release might influence trypanosome growth and the severity of infection.

Focusing on the early stage of trypanosome infection in the mammalian host, the effect of tsetse fly salivary components on parasitemia onset and on the involved host antiparasite immune effectors has been poorly investigated. Studies with other blood-sucking arthropods, such as ticks and sand flies, have demonstrated that salivary proteins are potent modulators of host innate and adaptive immune responses. Especially for ticks, which are characterized by their extended feeding time, a broad repertoire of immune modulatory activities has been described. These modulatory activities include the predominant induction of a Th2 response with an overall inhibition of proinflammatory and Th1 cytokines (23, 24, 38, 54), suppression of the effector functions of antigen-presenting cells (APCs) (5, 26, 28, 59), and modulation of T-cell (28, 59) and B-cell (17, 18) responses, as well as the inhibition of granulocyte infiltration (39, 53) and NK-mediated cytotoxicity (25). Illustrating the importance of salivary components for pathogen transmission, the tick protein Salp15 was shown to significantly increase the infectivity of *Borrelia burgdorferi* spirochetes in mice (50). Also, previous studies indicate that salivary extracts from Old and New World sand flies modulate several effector functions of the host immune system, promoting the initial *Leishmania* infection at the inoculation site (3, 16, 32, 58, 60).

A general immunological effect observed in several studied vector-host interaction models is an anti-inflammatory action of the salivary components and the occurrence of a Th2-associated cytokine response in exposed hosts (23, 24, 37, 38, 54). Consistent with this, we have previously demonstrated that tsetse fly saliva also biases the immune system to a Th2 response (4), possibly reflecting the anti-inflammatory potential of tsetse fly saliva. As a proinflammatory response in an early stage of trypanosome infection has been shown to be crucial for efficient control of parasitemia (21, 33, 44), a putative anti-inflammatory property of tsetse fly saliva might enhance trypanosome progression at the early stage of infection. Moreover, tsetse fly saliva was shown to suppress IgG responses.
against the heterologous antigen ovalbumin. (4) As such, the salivary components might also influence the host antitypanosome antibody induction responsible for parasitie clearance. This paper describes the effect of Glossina morsitans morsitans saliva on the onset of a Trypanosoma brucei brucei infection in mice and the associated immune responses.

MATERIALS AND METHODS

Animals. Six- to 8-week-old female Toll-like receptor-2/4 (TLR-2/4)-deficient mice (Jackson Laboratory, Bar Harbor, ME) were used for the experimental infections. Six- to 8-week-old female in-house-bred (BALB/c × C57BL/6) mice were used for antisaliva immunization and natural trypanosome infection via tsetse flies. Mouse care and experimental procedures were performed under approval from the Animal Ethical Committee of the Vrije Universiteit Brussel. Tsetse flies (Glossina morsitans morsitans) were available from the insectaria at the Prins Leopold Institute of Tropical Medicine Antwerp (ITMA) and originated from puparia collected in Kariba (Zimbabwe) and Handeni (Tanzania). Flies were fed on rabbits and maintained at 26°C and at a relative humidity of 65%. Animal ethics approval for the tsetse fly feeding on live animals was obtained from the Animal Ethical Committee of the Institute of Tropical Medicine, Antwerp (Belgium).

Parasites. Plectomorphic AnTat1.1E Trypanosoma brucei brucei parasites were used for the experimental infections done by use of an intradermal injection method. Parasites were grown in mice and purified from their hemiparalized blood by using DEAE-cellulose (DE52; Whatman) anion-exchange chromatography (27). Parasites were collected in PSG buffer (phosphate-buffered saline [PBS]; pH 7.4) supplemented with 10% glucose), centrifuged at 850 × g, and resuspended in sterile PBS or RPMI 1640 prior to injection. For natural infection experiments, tsetse flies were infected with the AnTAR1 Trypanosoma brucei brucei parasite strain, which was shown to efficiently develop into a mature salivary gland infection.

sVSG purification. The soluble form of the glycosylphosphatidylinositol−anchored VSG was purified from isolated AnTat1.1E parasites following a stress-induced activation (50 mM NaH2PO4, pH 5.5) of the endogenous phospholipase matocytometer in 1/200-diluted blood samples taken from the tail vein. Trypanosoma brucei brucei parasites were grown in mice and purified from their hemiparalized blood by using DEAE-cellulose (DE52; Whatman) anion-exchange chromatography (27). Parasites were collected in PSG buffer (phosphate-buffered saline [PBS]; pH 7.4) supplemented with 10% glucose), centrifuged at 850 × g, and resuspended in sterile PBS or RPMI 1640 prior to injection.

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Saliva affects systemic cytokine transcription and translation during infection. Systemic cytokine responses were analyzed in the blood at the transcriptional and translational levels after 3 and 6 days of infection, corresponding, respectively, to the early and progressed stages of systemic parasitemia (Fig. 1A). Cytokine transcription was assessed using RT-QPCR on RNA extracts from the WBC fraction (Fig. 3A). Several transcripts encoding type I and type II cytokines were significantly upregulated during infection compared to the naive condition. IL-6 and IL-12p35 transcripts, which were suppressed by saliva at the dermal infection site within a few hours of infection (Fig. 2), were not influenced systemically at days 3 and 6 postinfection (p.i.) (Fig. 3A). Although infections initiated with saliva resulted in a significantly more severe parasite burden, systemic transcription of several inflammatory cytokines by WBC was reduced in the early stage of infection. TNF mRNA (days 3 and 6 p.i., P < 0.05) and IL-10 mRNA (day 3 p.i., P < 0.05; day 6 p.i., P < 0.01) levels were lower before the first parasitemia peak upon infection with saliva. Transcripts encoding IFN-γ (day 3 p.i., P < 0.05; day 6 p.i., P < 0.1) and iNOS (day 6 p.i., P < 0.1) also tended to be suppressed.

The reduced TNF mRNA levels upon saliva-facilitated infection were also associated with lower serum TNF concentrations (day 3 p.i., P = 0.17; day 6 p.i., P < 0.1) (Fig. 3B). The transcriptional and translational differences for TNF coincide with the increased early outgrowth of the parasite population. IL-10 and IFN-γ concentrations were not affected in the serum.

Saliva does not inhibit antitypansome antibody titers. Antibody induction against the major membrane component of the infecting trypanosome clone, AnTat1.1E VSG, was analyzed in the early stage of trypanosome infection with or without tsetse fly saliva. IgM was the only AnTat1.1E sVSG-reactive antibody isotype that was significantly induced in both experimental groups prior to the first peak of parasitemia, as measured in ELISA at day 6 postinfection (Fig. 4). Associated with the higher parasite concentrations observed in the saliva-facilitated infection, an increased anti-sVSG IgM antibody ti-
Repetitive saliva exposure promotes the onset of trypanosome infection.

To evaluate the effect of repeated confrontation with saliva and saliva-induced immune responses on the onset of a trypanosome infection, mice were preexposed to repetitive tsetse fly bites. This preexposure resulted in an accelerated onset of a subsequent AnTAR1 infection initiated by the bite of a tsetse fly (day 6 p.i., $P < 0.01$; day 7 p.i., $P < 0.05$) (Fig. 5A). Consistent with the natural exposure, experimental immunization of mice against tsetse fly saliva also resulted in a significantly earlier onset of parasitemia upon natural trypanosome challenge in comparison to what was seen for control mice (day 6 p.i., $P < 0.01$; day 7 p.i., $P < 0.1$; day 8 p.i., $P < 0.05$) (Fig. 5B). Immunogenicity of saliva components was analyzed by Western blotting and ELISA, and it was shown that saliva-specific antibodies, including IgE isotypes, were raised upon exposure to saliva as described earlier (4). Post-first peak parasite concentrations in the blood and survival times of infected mice were not different in the different experimental groups.

**DISCUSSION**

The interaction of African trypanosomes with the host immune system is complex and involves several aspects, such as the antigenic plasticity of the parasite as well as the modulation of immune responses. In order to maintain controlled growth, trypanosomes coordinately alter their major surface antigen (VSG) coat and modify functions of antigen-presenting cells (8, 43) as well as T lymphocytes (56) and B lymphocytes (2). During a trypanosome infection, an equilibrated balance between inflammation and anti-inflammation was shown to be crucial for successfully controlling parasites while limiting the outcome of immune pathology (33, 44). Information on the immunology of African trypanosomiasis is available mainly from experimental trypanosome infections that exclude the vector-host interaction. The presented data demonstrate that tsetse fly salivary components play a significant role in the early infection stage by accelerating the early outgrowth of the trypanosome population. This effect did not result from differences in infectious dose or improved trypanosome viability in a saliva environment. As such, the faster onset of the trypano-
some infection could be the result of the interference of tsetse fly saliva with host hemostatic reactions (e.g., vasoconstriction) and host immune reactions, such as the recruitment and activation of host immune cells or the release of inflammatory mediators at the site of infection.

Vasoactive salivary factors, present in several blood-sucking arthropods (40, 52), might be responsible for increasing the parasite's efficiency in invading the lymphatic and blood vessels. In the case of *Plasmodium*, mosquito-mediated transmission resulted in an increased efficiency of the parasite to leave the dermal infection site compared to an experimental syringe injection (1). Moreover, the *Lutzomyia* sand fly promotes *Leishmania* transmission by the presence of the vasodilatory peptide maxadilan, with immune modulatory properties, in the saliva (29, 40, 41). In our study, trypanosome parasites reached earlier detectable levels in the bloodstream in the saliva-injected mice, indicating that salivary components might enhance vessel invasion from the dermal infection site.

Since trypanosomes are extracellular parasites, they are in principle more sensitive to antibody-mediated immune effects, including complement-mediated lysis (13, 46) and phagocytosis of opsonized parasites (9, 55). Although the involvement of antibodies in parasite clearance during an early stage of infection might be limited, the influence of tsetse fly saliva-facilitated infection on trypanosome-specific antibody induction was assessed by evaluating the levels of sVSG-specific antibodies in sera of infected mice. The higher parasite burden in the mice coinjected with saliva and trypanosomes is associated with higher titers of AnTat1.1E sVSG-specific IgM and IgG2a antibodies, most probably reflecting the increased confrontation of the host immune system with the trypanosomal VSG rather than a systemic saliva effect. The high VSG-specific antibody titers upon saliva-facilitated infection compared to those of the control infection might explain the equally efficient clearance of the first parasitemia peak despite the more severe parasite burden. Collectively, the infection-accelerating effect of saliva is not dependent on the inhibition of VSG-specific antibody induction.

Modulation of effector functions of APCs is another general feature of several arthropod salivary gland extracts. For instance, ticks and sand flies suppress the production of cytokines, NO, and reactive oxygen intermediates by APCs (26, 45, 59) and thereby might be involved in suppressing antipathogen responses. A major trypanosome component reported to trigger APCs is the VSG, in particular its glycosylphosphatidyl-inositol anchor. This molecule can trigger inflammatory TNF, IL-6, and IL-12 release from antigen-presenting cells through MyD88-dependent signaling, indicating an interaction with pattern recognition receptors (11). Within 3 hours of intradermal *T. brucei brucei* infection, local transcription of inflammatory genes was induced at the dermal site. Initiation of infection in the presence of tsetse fly saliva significantly suppressed local transcription of genes encoding IL-6 and IL12p35. Transcripts encoding other inflammatory molecules, such as TNF, IFN-γ, and iNOS, tended to be upregulated in the ear dermis in the early infection stage (1 to 3 h) but were not significantly inhibited by saliva. Systemically, reduced transcription of IFN-γ, TNF, and IL-10 in white blood cells was observed after 3 and 6 days of trypanosome infection initiated with saliva. Additionally, reduced TNF mRNA levels in the WBC compartment were corroborated by altered serum TNF concentrations at days 3 and 6 postinfection as measured by ELISA. As TNF was shown to exert trypanolytic activity for the AnTat1.1 *T. brucei* stock and to be directly involved in trypanosome control (33), the observed suppression correlates with the increased parasite burden upon saliva-facilitated infection. Together, these data indicate that the infection-promoting effect of tsetse fly saliva is associated with a suppression of local
inflammatory responses (IL-6 and IL12p35 mRNA) as well as a systemic effect on TNF production. Here, the systemic effect might result from the direct translation of the local anti-inflammatory action of saliva and, indirectly, from the higher parasite burden or from a combination of both. However, as the affected cytokines are produced mainly by APCs in response to trypanosome antigens, our observations suggest that the saliva-facilitated parasite onset modulates the activation of these cells in the early infection stage.

Besides affecting the responsiveness of APCs to trypanosome trypanosome components, tsetse fly saliva might inhibit APC activation by interfering with inflammatory triggers released upon tissue damage at the dermal site of infection. Based on the molecular identification of salivary components and suggestive functional information, tsetse fly saliva might interfere with ATP-mediated triggering of purinergic receptors on APCs and the subsequent induction of inflammatory cytokines (10). In this context, the enzymatic degradation of extracellular nucleotides has been proposed as a mechanism of anti-inflammation exploited by both endo- and ectoparasites, including blood-sucking arthropods (15, 51). Tsetse fly saliva has been demonstrated to contain components with apyrase (36), putative 5’-nucleotidase (GenBank accession no., AAK63848), and adenosine deaminase (31) activity. With this array of enzymes, saliva could convert a potential inflammatory nucleotide (ATP) to anti-inflammatory degradation products, such as adenosine and inosine (19, 20).

Another important immunological aspect of the vector-host interaction is the occurrence of hypersensitivity reactions at the site of the bite that might result from repetitive exposure to insects. In the case of sand flies, saliva enhances the severity of Leishmania infection, while delayed-type hypersensitivity (DTH) responses, raised by repeated exposure to the vector (22, 57) or immunization against individual salivary components (41, 60), can abrogate the Leishmania infection transmitted by the sand fly. Tsetse fly saliva also promotes the onset of trypanosome infection, while repeated exposure to tsetse fly bites induces saliva-specific IgE antibodies that might be involved in local hypersensitivity reactions (4, 12). Moreover, DTH responses have been observed earlier for tsetse fly-exposed rabbits (12). To evaluate the effect of repeated confrontation with tsetse fly saliva on the onset of a naturally transmitted trypanosome infection, mice were repeatedly exposed to tsetse feeding and immunized against total saliva in adjuvant. Upon challenge with infected tsetse flies, the onset of infection was faster in exposed and saliva-immune mice than in control mice. This indicates that antasilva immune responses can be beneficial for the initiation of trypanosome infection. This differs from what is seen for Leishmania transmission by the sand fly, where DTH responses against saliva or individual constituents can abrogate the infection (22, 41, 57, 60). However, the intracellular nature of the Leishmania parasite implies that the efficient penetration of APCs becomes a sensitive point in the onset of infection (47, 57), while trypanosomes as extracellular parasites appear unaffected by local hypersensitivity reactions (42) and might even benefit from the increased vasopermeability to leave the initial site of infection more efficiently. This does not exclude the possibility that other exposure schemes or immunization against individual salivary proteins might still result in protection against trypanosome transmission by tsetse flies. However, the presented saliva exposure experiments did not confer protective immunity against natural trypanosome challenge.

Together, the presented data demonstrate that tsetse fly saliva exerts an immune modulatory effect in the murine host associated with an infection-promoting effect during the early stage of a T. brucei infection. Moreover, repeated exposure to saliva can make mice more sensitive to infection in the early stage. Evaluation of the possible epidemiological importance of both effects would depend on an extensive experimental assessment of the minimal infective dose as a crucial parameter.

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