MOLECULAR CROSSTALKS IN *LEISHMANIA*-SANDFLY-HOST RELATIONSHIPS

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Summary:

Sandflies (Diptera: Phlebotominae) are vectors of *Leishmania* parasites, causative agents of important human and animal diseases with diverse manifestations. This review summarizes present knowledge about the vectorial part of *Leishmania* life cycle and parasite transmission to the vertebrate host. Particularly, it focuses on molecules that determine the establishment of parasite infection in sandfly midgut. It describes the concept of specific *versus* permissive sandfly vectors, explains the epidemiological consequences of broad susceptibility of permissive sandflies and demonstrates that genetic exchange may positively affect *Leishmania* fitness in the vector. Last but not least, the review describes recent knowledge about circulating antibodies produced by hosts in response to sandfly bites. Studies on specificity and kinetics of antibody response revealed that anti-saliva IgG could be used as a marker of host exposure to sandflies, i.e. as a useful tool for evaluation of vector control.

KEY WORDS : *Leishmania*, sandfly, host.

The leishmaniases are parasitic diseases with various clinical signs and symptoms ranging from skin lesions to life-threatening visceral disease. The etiological organisms, parasitic protozoa of the genus *Leishmania*, are transmitted by insect vectors, female phlebotomine sandflies (Diptera: Phlebotominae). During the life cycle, *Leishmania* adapt themselves to varied and heterogeneous environments of the insect vector and vertebrate host that differ in temperature, pH and many other parameters. Recent research revealed that the parasite development is affected by a number of molecules derived from the vector. This short review aims to demonstrate how molecular biology helps us to understand *Leishmania* epidemiology and the circulation of the parasite in nature. The text is divided into three parts. The first briefly summarizes the present knowledge about the life cycle of *Leishmania* in the vector and the other two deal with selected aspects of the *Leishmania*-sandfly-host interaction about which much new information has been gained during last few years.

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LEISHMANIA LIFE CYCLE IN THE SANDFLY

In the vector, parasites taken up with the blood meal undergo a period of replication and development in the midgut (reviewed by Molyneux & Killick-Kendrick, 1987), after which they differentiate to an infective metacyclic stage adapted for transmission to mammals (reviewed by Killick-Kendrick, 1990; Kamhawi, 2006). Initially, a blood meal containing aflagellated amastigotes is quickly surrounded by peritrophic matrix, a chitinous framework with a protein-carbohydrate matrix that is secreted by epithelial cells of the midgut (Pimenta *et al.*, 1997). Amastigotes divide and then differentiate into flagellated promastigotes, so-called procyclics. During this early stage of development, transforming parasites survive midgut proteases (Schlein & Romano, 1986; Dillon & Lane, 1993; Pimenta *et al.*, 1997; Schlein *et al.*, 1998; Ramalho-Ortigao *et al.*, 2003) and antimicrobial peptides (Boulanger *et al.*, 2004). The peritrophic matrix decays within a few days, the promastigotes escape from the endoperitrophic space and transform to long nectomonad stages. In the subgenus *Leishmania* (Suprapylarian species), promastigotes then attach by their flagella to the microvilli of the sand fly midgut (Killick-Kendrick *et al.*, 1974; Walters *et al.*, 1993; Cihakova & Volf, 1997; Warburg *et al.,* 1989; Sacks *et al.,* 2000). This attachment is an essential part of the *Leishmania* life cycle as it enables the parasite to avoid expulsion from the gut when the remnants of the digested blood meal are defaecated. The nectomonads multiply repeatedly in the abdominal midgut and gradually migrate forwards to the thoracic midgut and the stomodeal valve. These forms are the precursors of metacyclic promastigotes, small, highly motile forms with a long flagellum, which are infective for the vertebrate host (for recent review see Bates, 2007).

The stomodeal valve is situated between the midgut and oesophagus and ensures one-way flow of the food. During the late-stage infection, parasites cause pathological changes in the gut that facilitate the transmission to the vertebrate host (reviewed by Schlein, 1993; Bates, 2007). *Leishmania* block the anterior mid-

gut by the production of a viscous gel-like plug (promastigote-secretory gel-PSG) containing filamentous proteophosphoglycan (Ilg *et al.*, 1996; Stierhof *et al.*, 1999; Rogers *et al.*, 2004). In addition, they attach to the chitinous lining of the stomodeal valve and destroy this lining by chitinase (Schlein *et al.*, 1992, 1993). Unique filamentous structures connecting the lining with the apical end of the epithelial cells are also damaged (Volf *et al.*, 2004), supposedly by proteases. Because of the damaged valve and occlusion of the anterior midgut by PSG, the heavily infected sand fly female finds it difficult to take a blood meal and regurgitates metacyclic promastigotes into the skin of the vertebrate. In addition, infected females probe repeatedly increasing the chance of transmission (Killick-Kendrick *et al.*, 1977).

In various parasite-vector combinations, metacyclic promastigotes were repeatedly found in proboscis (for review see Molyneux & Killick-Kendrick, 1987) and deposition of these parasites in the skin during the next bloodfeeding seems to be the second important mode of *Leishmania* transmission. Moreover, in some parasite-vector combinations, motile metacyclic parasites were observed also in salivary glands (e.g. Killick-Kendrick *et al.*, 1996) and sandfly urine (Sadlova & Volf, 2001) raising questions about alternative ways of transmission (Bates, 2007).

Leishmania are inoculated into the vertebrate host together with saliva of the sandfly. Saliva contains a number of molecules with vasodilatory, anticoagulatory or anti-inflammatory properties. These molecules are important for blood feeding but also play a crucial role in *Leishmania* transmission (Titus & Ribeiro, 1988). Saliva modulates the immune response of a naive host enabling the establishment of *Leishmania* infection (reviewed by Rohousova & Volf 2006). On the other hand, repeated exposure to sandfly bites elicits an immune response that confers protection against naturally transmitted *L. major* Yakimoff & Schokhor in mice (Kamhawi *et al.*, 2000). Two molecules of sand fly saliva, vasodilatory peptide maxadilan of *Lutzomyia longipalpis* Lutz & Neiva and protein SP15 of *Phlebotomus papatasi* Scopoli, have been successfully used in mice as a transmission-blocking vaccines (Morris *et al.*, 2001; Valenzuela *et al.*, 2001). Sandfly species, however, differ in salivary proteins/peptides and their pharmacological and antigenic properties (Warburg *et al.*, 1994; Volf & Rohousova, 2001; Cerna *et al.*, 2002; Anderson *et al.*, 2006) and this variability may have an impact on the clinical manifestations of a *Leishmania* infection. In sibling species of the *Lu. longipalpis* complex, Warburg *et al.* (1994) suggested that the amount of the peptide maxadilan plays a crucial role in visceralization of *L. infantum* Nicolle (syn *L. chagasi* Cunha & Chagas). In addition, due to differences of salivary antigens between various sandfly species, the protective effect they confer is species-specific: mice immunized with *Lu. longipalpis* saliva were more resistant to *Leishmania amazonensis* Lainson & Shaw co-inoculated with saliva of this species but not to those co-inoculated with saliva of *P. papatasi* or *P. sergenti* Parrot (Thiakaki *et al.*, 2005).

MIDGUT MOLECULES DETERMINE THE ESTABLISHMENT OF *LEISHMANIA* INFECTION

Laboratory studies examining the development of different *Leishmania* in a range of vectors showed that sandflies fall into two groups. Several sandfly species are specific vectors as they display remarkable specificity for the *Leishmania* species they transmit. For example, *P. papatasi* supports the development of only *L. major* but not other parasite species tested (Killick-Kendrick *et al.*, 1994; Pimenta *et al.*, 1994). Another example of a specific vector is *P. sergenti*, the vector of *L. tropica* Wright (Killick-Kendrick *et al.*, 1995; Kamhawi *et al.*, 2000). In contrast, most other sandfly species examined to date support the development of a broad range of *Leishmania* species and fall into the second group called permissive vectors (Volf & Myskova, 2007). These include *Phlebotomus* species transmitting parasites of the *L. donovani* complex (Pimenta *et al.*, 1994; Myskova *et al.*, 2007) and *Lu. longipalpis*, the New World vector of *L. infantum* (Walters *et al.*, 1993). Evidently, the parasites are able to develop in any permissive sand fly species, if given the opportunity.

Studies with *L. major* in *P. papatasi* showed that the attachment is controlled by species-specific modifications of the major surface glycoconjugate of *Leishmania* promastigotes, lipophosphoglycan (LPG) (Kamhawi, 2006; Pimenta *et al.*, 1992; Pimenta *et al.*, 1994; Sacks *et al.*, 1995; Butcher *et al.*, 1996) that selectively binds to the midgut galectin receptor PpGalec (Kamhawi *et al.*, 2004; Kamhawi, 2006). In other *Leishmania*-sandfly pairs, the role of LPG in attachment has not been investigated in such detail and mechanisms underlying this broad permissivity of other sandfly species have not been elucidated. A variety of candidate molecules have been proposed to mediate this process, such as a relatively conserved flagellar protein (Warburg *et al.*, 1989).

Recent studies revealed that attachment of *Leishmania* promastigotes in permissive species correlates with the presence of O-glycosylated epitopes on the luminal midgut surface (Myskova *et al.*, 2007; Volf & Myskova, 2007) which may serve as binding sites for lectin-like components found on the surface of parasites. Successful development of LPG-defective mutants in per-

missive sandfly species showed that the establishment of different *Leishmania* in permissive vectors does not arise from interactions of LPG with sandfly lectins. Instead, O-glycoproteins localized on the microvillar border of the midgut are involved in a novel mechanism of attachment as they bind to *Leishmania* promastigotes (Myskova *et al.*, 2007). The "adherence paradigm" described in the *P. papatasi-L. major* combination seems to be inverted in permissive species: N-acetylgalactosamine (GalNAc) containing glycoproteins of the sandfly react with a parasite lectin-like receptor. Potential candidates for this receptor are heparin-binding proteins and lectin-like molecules reported previously in various *Leishmania* species, some of which occur on the cell surface and bind GalNAc (Hernandez *et al.*, 1986; Mukhopadhyay *et al.*, 1989; Kock *et al.*, 1997; Svobodova *et al.*, 1997).

The presence of a conserved sandfly GalNAc ligand-parasite interaction in several permissive sandfly vectors explains successful adaptation of *Leishmania* to sandflies other than the specific ones. An important example is the introduction of *L. infantum* from the Mediterranean to Latin America (Killick-Kendrick *et al.*, 1980; Mauricio *et al.*, 2000). In Southern Europe, this parasite is transmitted to dogs and humans by the permissive vector *P. perniciosus* Newstead and related species of the subgenus *Larroussius* (Killick-Kendrick, 1999). When European colonists arrived with their dogs in Latin America, the parasite was able to switch to a new permissive vector, *Lu. longipalpis* (Killick-Kendrick *et al.*, 1980). Similar phenomena may underlie transmission cycles of atypical *L. tropica* strains. The ecology of emerging cutaneous leishmaniasis caused by *L. tropica* was studied in two adjacent foci in northern Israel (Jacobson *et al.*, 2003; Svobodova *et al.*, 2006). In both foci rock hyraxes (*Procavia capensis*) served as reservoirs. Hyraxes are susceptible to *L. tropica* and experimentally infected hyraxes were shown to be infective to feeding *P. (Adlerius) arabicus* Theodor and *P. sergenti* (Svobodova *et al.*, 2006). However, these two foci in Israel differed with respect to both parasite strains and vector species. The LPG of *L. tropica* from the northern focus was characterized by abundant terminal β-galactose residues on the side chains. On the other hand, LPG side chains of other *L. tropica* isolates are mostly capped with glucose (Soares *et al.*, 2004). Due to LPG modifications, this strain of *L. tropica* is able to develop only in the O-glycosylated midgut of the local permissive vector, *P. arabicus*, but not in the specific vector, *P. sergenti* (Svobodova *et al.*, 2006).

Another recent study that supports the hypothesis about the role of LPG in the attachment of *L. major* to *P. papatasi* midgut was performed on natural genetic hybrids between *L. infantum* and *L. major*. While all *Leishmania* strains tested (*L. major, L. infantum* and two hybrid strains) developed well in the permissive vector *L. longipalpis,* only *L. major* and the hybrid strains produced late-stage infections in *P. papatas*i. In contrast, *L. infantum* was defaecated from the midgut with the blood meal remains and did not develop further (Volf *et al.*, 2007). Indirect immunofluorescence showed that hybrids possess a certain level of *L. major*-type LPG which thus appears to enable the attachment of *P. papatasi* to the midgut (Volf *et al.*, 2007). The finding of the ability of *Leishmania* hybrids to develop in *P. papatasi* may have important epidemiological implications. This sandfly is peridomestic, antropophilic and widespread in much of southern Europe, North Africa and western Asia. The finding that *L. infantum/ L. major* hybrids develop heavy late stage infections in *P. papatasi* suggests that hybrid strains could circulate using this sandfly vector.

In addition, these experiments with hybrids rise questions about genetic exchange in *Leishmania*. Sexuality and genetic exchange remain an elusive point in this parasite. *Leishmania* have developed asexual mechanisms for generating a large repertoire of genotypes and these asexual mechanisms are believed to contribute efficiently to parasite fitness (reviewed by Victoir & Dujardin, 2002) and some authors postulated that parasites succeed better without sex (Ayala, 1998). However, our study showed that fitness of *L. infantum/ L. major* hybrids increased when compared with *L. infantum*. Genetic exchange enabled hybrid parasites to survive in the specific vector *P. papatasi* which means that sex positively affected parasite fitness.

ANTIBODIES AGAINST SANDFLY SALIVA: MARKER OF EXPOSURE TO SANDFLIES AND RISK MARKER OF *LEISHMANIA* TRANSMISSION

Repeated exposure to sandfly bites elicits cellular and humoral immune responses in the host (Barral *et al.*, 2000; Valenzuela *et al.*, 2001; Volf & Rohousova, 2001; Rohousova *et al.*, 2005; Gomes *et al.*, 2006). This immune response to salivary antigens was found to be species-specific. Sera of animals bitten by *P. papatasi* did not cross-react with saliva of other *Phlebotomus* species tested (Volf & Rohousova, 2001). Similarly, sera of humans bitten by *P. papatasi* and *P. sergenti* did not cross-react with *Lu. longipalpis* salivary antigens (Rohousova *et al.*, 2005).

Recent findings have stimulated interest in the use of anti-sandfly saliva antibody diagnostics as a novel tool for measuring exposure risk of *Leishmania* transmission among humans living in endemic regions. In such regions, host exposure to sandflies is an important epidemiological factor which could be used to estimate

the risk of parasite transmission and to measure the effect of insecticide campaigns on sandfly population size. Although methods of measuring the mass effect of control campaigns on sandfly survival and population size have been established (CDC traps, sticky traps) (Lewis & Ward, 1987), these cannot be employed in the field to determine individual protection of the host. Moreover, such a tool will be useful also for epidemiological analysis of risk factors.

To assess the feasibility of employing anti-saliva antibody response in vector exposure screening programmes, we studied the kinetics of anti-saliva antibody response in dogs experimentally exposed to *Lu. longipalpis*. Anti-saliva IgG and its subclasses IgG1 and IgG2 were found as useful markers of exposure to sandflies in experimentally exposed dogs as they reflected the intensity of exposure (numbers of bloodfed *Lu. longipalpis* females). Increased antibody levels and differences between high- and low-exposed dogs were detectable throughout the study, *i.e.* more than six months after the last exposure (Hostomska *et al.*, in press). In many foci of leishmaniasis, sandfly populations show seasonal fluctuations or sandfly-free periods and our results suggest the antibodies can persist until the next sandfly season. Screening of dog sera for specific IgG against salivary antigens of the vector was therefore suggested as a useful epidemiological tool in visceral leishmaniasis foci. Together with infection incidence monitoring, such data would provide valuable information on control programme effectiveness.

There are, however, two main obstacles to routine use of anti-saliva antibody screening . Dissection of large number of salivary glands is time-consuming, laborious, and it requires productive sandfly colonies. In addition, the number of different antigens in whole salivary gland homogenate increases the chance of nonspecific reactions, since some antigens are shared between different sandfly species (Volf & Rohousova, 2001; Rohousova *et al.*, 2005). To address these problems, a robust method for identification of immunodominant salivary proteins and their production as recombinant proteins was described (Valenzuela, 2002). Recently, seven recombinant salivary proteins from *L. longipalpis* were tested with human and fox sera from an area endemic for visceral leishmaniasis and with sera of dogs experimentally exposed to sandflies (Gomes *et al.*, 2007a). Some proteins were recognized by all tested sera which indicates the use of recombinant salivary proteins as potentially feasible epidemiological tool for evaluation of the exposure.

As anti-sandfly saliva antibodies are a good marker of exposure to sandfly bites, their possible use as a marker of risk of *Leishmania* transmission was tested. In an analogous setting, anti-vector saliva antibody response was suggested as a marker of risk of malaria or Lyme disease transmission (Remoue *et al.*, 2006; Schwartz *et al.*, 1991). However, in humans from endemic area for visceral leishmaniasis in Brazil, the presence of antibodies against *Lu. longipalpis* salivary antigens strongly correlated with the development of anti-*Leishmania* delayed type hypersensitivity (a marker of protection) while individuals that converted for anti-*Leishmania* antibodies did not recognize any sandfly salivary proteins (Gomes *et al.*, 2002). On the other hand, a positive correlation between infection and anti-saliva antibody titres was found in foci of cutaneous leishmaniases caused by *L. tropica* and *L. major* (Rohousova *et al.*, 2005; Louzir *et al.*, 2005) in Turkey and Tunisia, respectively. In Turkey, human sera were collected in an endemic focus of *L. tropica* where *P. sergenti* is the vector, while *P. papatasi* is refractory to this *Leishmania* species. In comparison with non-infected individuals from the same place, patients with active lesions had stronger antibody response to saliva of the vector *P. sergenti* while no correlation was found with antibody response to the non-vectorial species *P. papatasi* (Rohousova *et al.*, 2005).

In conclusion, two independent studies on cutaneous leishmaniases caused by different *Leishmania* species revealed that antibodies against salivary proteins of the vector could be used as a marker of risk of *Leishmania* transmission. Studies on sandflies gave the first direct evidence that antibodies against saliva of the vector could give a measure of the contact between a vector and a human population in an endemic area and thus may provide an estimate of the risk of transmission of a vector-borne disease.

ACKNOWLEDGEMENTS

We are grateful to Professor R. Killick-Kendrick for valuable comments to the manuscript. Authors are supported by the Ministry of Education (MSM0021620828, LC06009), Czech Science Foundation (206/06/0967, 206/06/P015) and GAUK (139/2006/B-Bio/PrF, 191/2006/B-Bio/PrF).

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