The protective effect against Leishmania infection conferred by sand fly bites is limited to short-term exposure

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Under laboratory conditions, hosts exposed twice to sand fly saliva are protected against severe leishmaniasis. However, people in endemic areas are exposed to the vector over a long term and may experience sand fly-free periods. Therefore, we exposed mice long- or short-term to Phlebotomus duboscqi bites, followed by Leishmania major infection either immediately or after a sand fly-free period. We showed that protection against leishmaniasis is limited to short-term exposure to sand flies immediately before infection. Our results may explain the persistence of leishmaniasis in endemic areas and should be taken into account when designing anti-Leishmania vaccines based on sand fly saliva.

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Leishmania spp. are intracellular protozoan parasites which infect mammalian phagocytic cells. The clinical manifestations of their multiplication and associated immunopathology depend on parasite species, genetic background and immune status of the host, ranging from cutaneous to fatal visceral disease. Leishmania major is a typical zoonotic species causing the cutaneous form of the disease. The transmission occurs predominantly between the vector and wild rodents (e.g. sand rats) with humans occasionally breaking into the zoonotic transmission cycle (Peters and Killick-Kendrick, 1987). The sole vectors of Leishmania parasites are female sand flies (Diptera: Phlebotominae), tiny bloodsucking insects widespread in the subtropics and tropics. During blood feeding, the parasites are deposited into host skin together with the fly saliva. Sand fly saliva contains antihemostatic molecules and factors which modify the immune milieu of the skin. Not only do these components facilitate the acquisition of blood, but their presence in the infection site is also important for effective establishment of Leishmania parasites (Rohousova and Volf, 2006).

Individuals exposed to sand fly bites develop a specific immune response to salivary proteins (Rohousova and Volf, 2006). It was hypothesized that the enhancing effect of sand fly saliva on Leishmania infection could be abolished by this immune response in humans vaccinated against vector saliva, thereby preventing the establishment of infection. This concept has been demonstrated for Leishmania spp., causing both cutaneous and visceral leishmaniasis. Partial protection against L. major infection has been achieved in mice immunized by the bites of uninfected sand flies (Kamhawi et al., 2000), by salivary gland homogenate (Belkaid et al., 1998) or individual salivary components (Morris et al., 2001; Valenzuela et al., 2001; Oliveira et al., 2008). Protection has generally been associated with the production of IFN-γ and IL-12 upon challenge with Leishmania and saliva (Kamhawi et al., 2000; Gomes et al., 2008; Oliveira et al., 2008), suggesting that the existing anti-saliva delayed-type hypersensitivity (DTH) immune response creates an inhospitable environment for parasite survival.

While previous exposure of animals to sand fly feeding under laboratory conditions interferes with subsequent growth of transmitted Leishmania parasites, such protection has not been reported from the field. In endemic areas, the prevalence of Leishmania infection within the sand fly population is relatively low (Peters and Killick-Kendrick, 1987) and hosts are mostly exposed to the bites of uninfected sand flies. Despite this continuous exposure, leishmaniasis persists in endemic areas. Local inhabitants develop a specific antibody response to salivary antigens (Gomes et al., 2002; Rohousova et al., 2005; de Moura et al., 2007), which correlates with protection against visceral leishmaniasis (Gomes et al., 2002) but not against cutaneous leishmaniasis (Rohousova et al., 2005; de Moura et al., 2007). Clearly, the type of immune response elicited by laboratory immunization schemes and continuous exposure to uninfected sand flies occurring in the field are different.
Thus, in our study we developed a mouse model of natural sand fly exposure to compare the outcome of infection in mice immunized using the exposure scheme previously reported (Belkaid et al., 1998; Kamhawi et al., 2000; Thiakaki et al., 2005) and in mice exposed “naturally”, for a prolonged period.

Mice were bitten by Phlebotomus dubosci and subsequently infected by *L. major*, the sole *Leishmania* sp. naturally transmitted by this sand fly sp. (Peters and Killick-Kendrick, 1987; Killick-Kendrick, 1990). The exposure schemes used in our study also address the seasonal dynamics of sand fly populations (Peters and Killick-Kendrick, 1987) and the persistence of the humoral immune response to sand fly saliva throughout the sand fly-free period.

All animals used in this study were maintained and handled strictly in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24773/2008–10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic. Mice were anaesthetized by i.p. injection of 150 mg/kg ketamine and 15 mg/kg xylazine during the exposure phase and infection. All efforts were made to minimize the number and suffering of experimental animals within the study.

BALB/c mice were maintained in the animal facility of Charles University in Prague. Preliminary experiments were carried out to determine the optimal infection dose and exposure scheme. For the main study, 30 female mice (4 weeks old) were divided into five groups and exposed to *P. dubosci* females (colony originating from Senegal). At each exposure, 30 female sand flies were allowed to feed on whole mouse body (for exposure schemes see Fig. 1A) with an average of 27 fed sand flies per mouse. The mice were subsequently infected intradermally in the right ear pinna with $10^4$ *L. major* promastigotes (MHOM/IL/67/LRC-L137 JERICHO II) together with $1/4$ gland pair equivalent of *P. dubosci* salivary gland homogenate in 5 µl saline. Lesions were measured using a digital caliper. Seven weeks after the infection, mice were sacrificed and sampled for blood, infected ears and draining lymph nodes. In total, mice were followed for 37 weeks.

A group of *P. dubosci* saliva IgGs were measured by immunoblot and ELISA as previously described (Rohoušová et al., 2005). Immunoblots were performed on salivary gland homogenate separated by SDS–PAGE on 12.5% gel under non-reducing conditions. An equivalent of 50 gland pairs was loaded. After transfer, the membrane was cut into strips and incubated with mouse sera diluted 1:100 and goat anti-mouse IgG peroxidase-conjugate (heavy chain-specific, Sigma–Aldrich) diluted 1:750. For ELISA tests, wells were coated with *P. dubosci* salivary gland homogenate (1/40 gland pair equivalent), sera were diluted 1:200 and goat anti-mouse IgG peroxidase-conjugate diluted 1:1000. The same ELISA protocol was used to measure specific anti-*L. major* IgG with two minor modifications: wells were coated with crude *L. major* promastigotes (10⁶ cell equivalents per well) and sera were diluted 1:400.

Parasite burdens in the infected ear and draining lymph node were quantified by PCR–ELISA using a protocol described earlier (Kobets et al., 2010). The following primers were used: digoxigenin-labeled F 5′-ATT TTA CAC CAA CCC CCA GTT-3′ and biotin-labeled R 5′-GTG GGG GAG GGG CGT TCT-3′ (VBC-Genomics Biosciences Research, Austria). The cumulative parasite load was calculated as the sum of the parasite loads in both tested tissues.

Lesion size development was analyzed by general linear models (GLM) ANOVA and Scheffe’s Multiple Comparison Procedure after data transformation (ln(x+1)). Other data (parasite load and antibody production) were subjected to non-parametric Wilcoxon tests. For correlation tests we used the non-parametric Spearman Rank Correlation Matrix. Statistical analyses were performed using NCSS 6.0.21 software.

*Leishmania* infection was monitored in BALB/c mice exposed to *P. dubosci* bites following four different exposure schemes (Fig. 1A). Groups 2 and 15 represented short- and long-term exposures during the sand fly season with subsequent *Leishmania* transmission. Schemes used for groups 2 + 0 and 15 + 0 mimicked short- and long-term exposures followed by a sand fly-free period and *Leishmania* transmission occurring during the subsequent sand fly season.

In accordance with previous reports (Belkaid et al., 1998; Kamhawi et al., 2000; Thiakaki et al., 2005), *Leishmania* lesion size differed significantly between group 2 and control mice; in immunized mice the lesion size was smaller from week 3 p.i. onwards. Protection against *L. major* infection has previously been reported only for mice immunized by the saliva of *Phlebotomus papatasi* (Belkaid et al., 1998; Kamhawi et al., 2000). Here we report, to our knowledge for the first time, a similar protective effect in mice repeatedly bitten by *P. dubosci*, the other proven vector of *L. major* (Peters and Killick-Kendrick, 1987; Killick-Kendrick, 1990).

Although *L. major* established infection in immunized mice (group 2), the skin damage remained mild and the cumulative parasite load was significantly reduced by approximately threefold at week 7 p.i. (Fig. 1E). This finding corresponds with data published previously, where the number of parasites in *P. papatasi*-immunized mice was significantly reduced up to 8 weeks p.i. (Belkaid et al., 1998). The present study provides a model for further exploration of the vector-parasite-host immune interactions that influence establishment of *L. major* infection with mild or absent skin damage. Such a controlled amastigote population is expected to act as the sustained source of *L. major* being transmissible to another sand fly.

In some regions, sand fly populations vary greatly during the year, with sand flies occurring during several months of the year (Peters and Killick-Kendrick, 1987). To mimic this situation, mice of group 2 + 0 were exposed to *P. dubosci* bites twice, similar to group 2, but with a subsequent delay of 15 weeks before the challenge with *L. major*. Compared with group 2, these mice showed significantly larger lesion size (Fig. 1B and D) and greater parasite load (Fig. 1E). All parameters of infection were equal to the control, non-immunized mice (Fig. 1B,D,E), strongly indicating that the protective effect was lost in those mice.

Long-term exposure to sand flies with possible desensitization to salivary antigens is another scenario that is likely to occur in endemic areas and is not reflected in studies published to date. To simulate this situation, the last two groups of mice were immunized 15 times, either directly before infection (group 15) or with a subsequent delay of 15 weeks before the challenge (group 15 + 0). Similar to group 2 + 0, the protective effect against the development of infection measured by lesion size was completely abrogated in both long-term exposure groups (Fig. 1B), with parasite load being comparable with that found in the control group (Fig. 1E). In the group 15 + 0, the parasite load in the infected ear was significantly higher than in the protected group (Fig. 1E). To date, desensitization to blood feeding insect saliva has only been described in mosquitoes (Peng and Simons, 1998). Here we show that a similar phenomenon may also occur in sand fly-infested areas, but the underlying mechanism needs to be elucidated. We can hypothesize that immunization with a large antigen load tends to skew the immune system towards a Th2 response which, in the case of groups 15 + 0 and 15, could be associated with the lack of...
protection. Additionally, the severity of skin damage could be due to the deposition of immune complexes in the skin, leading to an exacerbated immune response from the early stage of lesion development. These immune complexes are more likely to form between anti-saliva antibodies and salivary proteins since no binding was observed between anti-\textit{P. duboscqi} saliva antibodies and \textit{L. major} promastigotes (Rohousová et al., unpublished data).

All groups of mice showed elevated anti-\textit{Leishmania} IgG at week 7 p.i. (Fig. 1F) with a positive correlation found between the levels of anti-\textit{L. major} IgG and the lesion size (Fig. 1C). Accordingly, only the protected group (group 2) had significantly lower anti-\textit{L. major} IgG levels compared with non-immunized mice (Fig. 1F), correlating well with the lowest parasite load in this group.

In areas where cutaneous leishmaniasis is endemic, the presence of active \textit{Leishmania} lesions in patients is associated with elevated
levels of anti-vector saliva IgG (Rohoušová et al., 2005; de Moura et al., 2007), suggesting that these antibodies could be used as a risk marker for Leishmania transmission. Accordingly, we measured anti- \textit{P. duboscqi} IgG levels and correlated those with the status of \textit{Leishmania} infection. At the end of immunization (week 30, before infection), only groups 15 + 0, 2 + 0 and 15 had elevated levels of anti- \textit{P. duboscqi} saliva IgG, with group 15 having the highest level (Fig. 2A) and strongly recognizing at least seven out of 12 protein bands within the broad range of 12–60 kDa (Fig. 2B). The immunoblot revealed that in group 15 + 0, the level of specific antibodies decreased similarly for all antigens. Mice in group 2 + 0 recognized only one or two antigens, with the protein band of approximately 42–45 kDa being the strongest antigen. Mice from groups 2 and control showed weak or no visible reaction with \textit{P. duboscqi} salivary proteins (Fig. 2B). Within all tested groups, a positive correlation was found between anti- \textit{P. duboscqi} IgG and \textit{Leishmania} lesion parasite load ($k = 0.38, P = 0.04$), supporting the above mentioned hypothesis that anti- \textit{P. duboscqi} IgG correlates with skin damage. On the other hand, no correlation was found between anti- \textit{P. duboscqi} IgG and parasite load in the draining lymph node, indicating that pre-exposure to \textit{P. duboscqi} bites could not alter parasite dissemination. Moreover, groups with a delay between pre-exposure and infection (groups 15 + 0 and 2 + 0) had significantly higher parasite loads in the ears than in the draining lymph nodes (Fig. 1E), indicating that in bitten hosts \textit{L. major} preferentially multiplies in the skin tissue.

The hypothesis that different immune regulatory mechanisms operate in those tissues is also supported with our previous study showing that parasite numbers in lymph nodes and development of skin lesions are under distinct genetic control (Kurey et al., 2009).

Anti-\textit{P. duboscqi} saliva antibodies were undetectable or absent throughout the study in two groups – group 2 and unexposed control mice (Fig. 2). Taking into account the outcome of infection in these groups, we assume that one of the following applies to mice negative for anti-saliva antibodies: (i) the host is sand fly saliva-naïve; therefore saliva in the infective inoculum would exacerbate the development of infection and accelerate skin damage, as in the control group, or (ii) the host has been recently immunized with a low dose of antigen, thus the antigen dose and/or the time-frame did not allow the production of detectable levels of specific anti-saliva antibodies. At the same time, the immune response resulting from this immunization scheme protects the host against leishmaniasis and although \textit{L. major} establishes an infection, the skin damage remains mild (as in group 2). This assumption is in agreement with field results from an endemic area of cutaneous leishmaniasis caused by \textit{Leishmania braziliensis}: low levels of anti-vector saliva IgG were found both in individuals without previous contact with \textit{Leishmania} and in individuals positive for anti-\textit{Leishmania} DTH, but apparently protected from lesion development (de Moura et al., 2007), possibly by short-term or low exposure to sand flies. A different situation has been reported from an endemic area of visceral leishmaniasis where anti-vector saliva antibodies in exposed individuals positively correlated with anti-parasite cell-mediated protective immunity (Gomes et al., 2002).

The concept of using sand fly salivary proteins in anti-\textit{Leishmania} vaccines is based on reports of Belkaid et al. (1998) and Kamhawi et al. (2000), and has been reinforced by several studies testing particular salivary proteins (Morris et al., 2001; Valenzuela et al., 2001; Vinhas et al., 2007; Gomes et al., 2008; Collin et al., 2009). This concept is not rejected by our study, since proteins administered as a vaccine may prime a different immune response from those naturally deposited by sand flies (Plotkin, 2005). Moreover, the targeted host species, humans and dogs, are outbred and therefore more diverse in terms of resistance and susceptibility to leishmaniasis than one strain of inbred laboratory mice.

Our study on BALB/c mice attempts to test the limitations of the sand fly saliva-induced protective effect on \textit{Leishmania} infection and several questions to be addressed were raised. (i) What is the kinetics of anti-saliva antibody and cellular immune responses? (ii) What is the mechanism underlying the loss of protective effect conferred by exposure to salivary antigens? Could antibodies block the protective effect as suggested by in vitro studies (Belkaid et al., 1998; Cavalcante et al., 2003)? (iii) Would saliva-induced protection be lost upon long-term exposure to sand flies even in other host species, e.g. mouse strains resistant to \textit{Leishmania} infection or dogs?

In conclusion we described here, to our knowledge for the first time, limitations of the sand fly saliva-induced protective effect on the development of \textit{Leishmania} infection. In previous studies, hosts protected by immunization with sand fly saliva (either by bite or by injection) were immunized twice at 1- or 2-week intervals and infected immediately thereafter (Belkaid et al., 1998; Kamhawi et al., 2000). Our results might help to explain the persistence of \textit{Leishmania} infection in endemic areas and should be taken into account when designing and testing vaccines based on vector salivary proteins.

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