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Original article

Sand fly specificity of saliva-mediated protective immunity in *Leishmania amazonensis*-BALB/c mouse model

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Abstract

Immune response of BALB/c mice to the salivary antigens of sand flies was found to vary with different species used, i.e. *Phlebotomus papatasi*, *Phlebotomus sergenti* and *Lutzomyia longipalpis*. Exposure of mice to bites of these sand flies elicits production of antibodies, which are largely specific to different saliva antigens previously identified as unique to the respective fly species. When immunized intradermally (i.d.) with salivary gland lysates (SGL) of *L. longipalpis*, BALB/c mice developed partial protective immunity against challenges in the contralateral ears with *Leishmania amazonensis* plus the gland lysates. Preimmunization of these mice with the lysates from the other two species was ineffective, further indicative of the specificity of saliva-mediated immune response. The partial protective immunity observed is significant, although it is not as dramatic as reported previously in a different sand fly-mouse model. There is a correlation of this immunity with a lower number of mononuclear and polymorphonuclear phagocytes at the site of parasite inoculation. Vector species-specificity of this immunity implies its elicitation by unique saliva antigen—an issue which requires attention when designing saliva-based vaccines against leishmaniasis.

Keywords: *Leishmania amazonensis*; Sand fly; Saliva; Protective immunity; *Phlebotomus*; *Lutzomyia*

1. Introduction

Saliva from two important sand flies, *Lutzomyia longipalpis* and *Phlebotomus papatasi*, which are hosts and vectors of *Leishmania*, are known to have immunomodulatory activities (reviewed in [1]). Co-injection of *Leishmania* with salivary gland lysates (SGL) or a recombinant salivary vasodilator, maxadilan, has been shown to enhance *Leishmania* infectivity [2]. Interestingly, recent data indicate that both parasite- and sand fly-derived factors contribute to this [3]. It also has been shown that mice are protected against *Leishmania major* when pre-exposed to non-infected sand flies, or pre-immunized with SGL or one of its components, such as *L. longipalpis* maxadilan and a *P. papatasi* 15 kDa protein [2,4–6]. This protection is thought to result from humoral immunity against salivary components alone or in combination with cell-mediated immunity manifested as delayed-type hypersensitivity (DTH) reaction at the inoculation site. Recent experimental data using the mouse model favor the latter possibility [2,6]. It has been reported that a similar immune response is induced in humans against saliva to produce anti-saliva antibodies and anti-*Leishmania* DTH response, which presumably contributes to the development of protective immunity against *Leishmania* infection [7].

The composition of salivary molecules as well as their functions and antigenicity seem to vary considerably among different sand fly species in both New World and Old World. This is true even among colonies of the same species of different geographical origin [8–12]. In endemic areas, it is likely that individuals at risk of exposure to *Leishmania*-harbouring sand flies are actually bitten more frequently by uninfected

Abbreviations: i.d., intradermally; SGL, salivary gland lysates.

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vectors of different species. It is therefore important to determine whether there are antigenic components common to all species relevant to this protective immunity against *Leishmania* relying on saliva-reactive immune effectors.

In the present study, we report that sera from mice experimentally bitten separately by *P. papatasi*, *Phlebotomus sergenti*, and *L. longipalpis* all contain anti-saliva antibodies, but they recognize largely antigens specific to the respective species. Results from separate experiments further showed that BALB/c mice were rendered less susceptible against *Leishmania amazonensis* infection when pre-immunized with SGL from *L. longipalpis*, but not with those from the other two species. Although none of the three sand fly species used in this study is the natural host or vector of *L. amazonensis*, *L. longipalpis* experimentally supports the full development of many *Leishmania* spp. [13], including *L. amazonensis* [14] and *L. mexicana* [15].

2. Materials and methods

2.1. Sand flies and their salivary gland lysates

Sand flies were reared as previously described [10]. Laboratory colonies of different genera (*Lutzomyia* versus *Phlebotomus*) and two species within the *Phlebotomus* genus, *P. papatasi* and *P. sergenti*, were used. The later two species were collected originally from Turkey in 1999. *L. longipalpis* originated from Jacobina, Brazil and was kindly provided by Professor Ward, Liverpool School of Tropical Medicine, in 1992. SGLs from 5 to 10-day-old females were dissected in Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.6) and stored at –70 °C. Before use, 20 salivary glands were disrupted in 20 μl of buffer by three cycles of freezing/thawing.

2.2. Leishmania parasites

L. amazonensis (LV78, MPRO/BR/72/M1845) promastigotes of a virulent clone 12-1 were freshly differentiated at 26 °C from footpad lesion-derived amastigotes of infected BALB/c mice and grown in Medium 199 (Biochrom, Berlin) supplemented with 10% heat-inactivated foetal bovine serum (Gibco, UK) and 10 mM HEPES (pH 7.4, Biochrom). The promastigotes were passaged in vitro once, harvested at stationary phase and used after washing.

2.3. Animals

Female BALB/c mice used were bred and maintained under pathogen-free conditions at Hellenic Pasteur Institute according to European Union guidelines and legislation for the care and use of animals for research purposes. Mice between 6 and 7 weeks of age used for the present studies were tested periodically for absence of viruses according to the IRB guidelines.

2.4. Immune sera from fly-bitten mice

Sera were collected from three groups of separately caged mice after anaesthetization (ketamin 150 mg/kg and xylazin 15 mg/kg, intraperitoneally). They were exposed for 1 h, weekly for 10 times over a period of ~3 months, to groups of ~50 females each of *P. papatasi*, *P. sergenti* and *L. longipalpis*, respectively. One week after the last exposure, mice were bled for serum collections. Pre-immune sera were also collected for use as controls.

2.5. Immunodot blot

Dot blots were performed using SGL dotted on nitrocellulose membrane (NC2, Serva) in 2 μl aliquots, corresponding to 1 gland per dot. The membrane was blocked with 5% low fat dried milk in Tris buffer with 0.1% Tween 20 (Tris–Tw) for 1 h and then incubated with mouse sera diluted 1:500 in Tris–Tw buffer for 1 h. After several washings, samples were incubated for 1 h with peroxidase-conjugated swine anti-mouse IgG (SwAM/Px, SEVAC, Prague) at 1:1000 in Tris–Tw. Reaction products were visualized after development with diaminobenzidine and H_2O_2.

2.6. Experimental inoculation of mice

Groups of mice were immunized intradermally (i.d.) [4] in their left ear, each with SGL from one of the three different sand fly species. Each mouse received one gland-equivalent SGL in 10 μl PBS twice at 2-week interval. Two weeks after the last immunization, mice were challenged i.d. in the right ear with 10^6 stationary phase promastigotes with SGL, equivalent to one *L. longipalpis* gland. Non-immunized controls consisted of two groups: mice inoculated i.d. in the right ear each with 10^6 stationary phase promastigotes alone and those similarly inoculated, together with SGL equivalent to one *L. longipalpis*-gland. The diameter of the induration in each ear lesion was measured weekly for 16 weeks by using a calliper.

2.7. Limiting dilution assay to assess parasite loads in infected tissues

Parasite load was determined using the quantitative limiting dilution assay as described by Titus et al. [16]. Briefly, infected ears were aseptically removed from individual mice at the completion of the experiments after 16 weeks. Tissues were homogenized and diluted in Schneider's insect cell culture medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated foetal bovine serum, 100 U of penicillin per ml and 100 μg/ml of streptomycin per ml. Homogenate samples were each serially diluted in microtiter 96-wells in quadruplets and incubated for one week at 26 °C. Wells with positive growth were noted at specific dilutions as a measure of the parasite burdens in the lesions [16]. Results were expressed as mean –log parasite titer ± S.D. [17].

2.8. Flow cytometry of cells recovered from ear tissues

Three mice were sacrificed per group 2 weeks after challenges for isolating leukocytes from the ear dermis. Ears were