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.

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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
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. papatasii, 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 many L. longipalpis SGL from Leishmania amazonensis that BALB/c mice were rendered less susceptible against 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. papatasii 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. papatasii, 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 H2O2.

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 10⁶ 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 10⁶ 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
individually collected, rinsed with 70% ethanol and processed as previously described [4]. Isolated cells were fixed with 2% paraformaldehyde for 10 min at 4 °C. Non-specific binding of antibodies to the Fe-receptors of these cells was minimized by treating them for 30 min at 4 °C with an anti-FcγIII/II receptor antibody (2.4G2; PharMingen, San Diego, CA) in PBS containing 1% BSA and 10% normal mouse serum. 10^5 cells were then reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, USA) were used as isotype controls for nonspecific reaction of the cells. Double labeling was obtained by simultaneous incubation of both antibodies. Monocytes were identified as F4/80 positive and MHC class II low or negative and neutrophils as NIMPR-14 positive [18]. All reagents were made up in serum-free medium. 10^5 cells were reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, San Diego, CA) in PBS containing 1% BSA and 10% normal mouse serum. 10^5 cells were then reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, San Diego, CA) in PBS containing 1% BSA and 10% normal mouse serum. 10^5 cells were then reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, USA) were used as isotype controls for nonspecific reaction of the cells. Double labeling was obtained by simultaneous incubation of both antibodies. Monocytes were identified as F4/80 positive and MHC class II low or negative and neutrophils as NIMPR-14 positive [18]. All reagents were made up in serum-free medium. 10^5 cells were reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, USA) were used as isotype controls for nonspecific reaction of the cells. Double labeling was obtained by simultaneous incubation of both antibodies. Monocytes were identified as F4/80 positive and MHC class II low or negative and neutrophils as NIMPR-14 positive [18]. All reagents were made up in serum-free medium. 10^5 cells were reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, USA) were used as isotype controls for nonspecific reaction of the cells. Double labeling was obtained by simultaneous incubation of both antibodies. Monocytes were identified as F4/80 positive and MHC class II low or negative and neutrophils as NIMPR-14 positive [18]. All reagents were made up in serum-free medium. 10^5 cells were reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, USA) were used as isotype controls for nonspecific reaction of the cells. Double labeling was obtained by simultaneous incubation of both antibodies. Monocytes were identified as F4/80 positive and MHC class II low or negative and neutrophils as NIMPR-14 positive [18]. All reagents were made up in serum-free medium. 10^5 cells were reacted with marker-specific monoclonal antibodies [4]. Staining of cells was done using the following antibodies: biotin-conjugated anti-F4/80 antibody (PharMingen, USA) were used as isotype controls for nonspecific reaction of the cells. Double labeling was obtained by simultaneous incubation of both antibodies. Monocytes were identified as F4/80 positive and MHC class II low or negative and neutrophils as NIMPR-14 positive [18]. All reagents were made up in serum-free medium. 10^5 cells were reacted with marker-specific monoclonal antibodies [4].

2.9. Statistical analysis

Student’s t-test was performed to determine the statistical significance of the data obtained.

3. Results

3.1. Exposure of BALB/c mice to sand flies of different taxa elicits genus- and species-specific anti-saliva antibodies

BALB/c mice exposed to female sand flies of different genera and species as described produced sand fly species-specific anti-saliva antibodies. In all cases, these antibodies reacted with homologous antigens; cross-species antigen-antibody reactivity, if any, was weak (Fig. 1) indicating that mice bitten by sand flies produce anti-saliva antibodies, which recognize mainly SGL antigens or antigenic epitopes.

3.2. Immunomodulating properties of L. longipalpis salivary gland lysates on the L. amazonensis infection of BALB/c mice

When L. longipalpis SGL were co-inoculated with L. amazonensis promastigotes using the ear dermis model [4], lesions developed more rapidly and were larger in size (Fig. 2A, open square) than those of control mice (P < 0.001) (Fig. 2A, solid square). When mice were pre-immunized with L. longipalpis SGL, ear lesions also developed after challenges with the parasites plus the homologous SGL, but they were significantly smaller in size and grew more slowly (Fig. 2A, solid triangle) than those of the control groups (Fig. 2A, open and solid squares). After 16 weeks at the end of the experiments, ear lesions were approximately half of size in L. longipalpis SGL pre-immunized group than those in the control groups (P < 0.001). The observed partial protection conferred by L. longipalpis saliva against L. amazonensis was specific to this sand fly species. Mice pre-immunized with SGL from other species, i.e. P. papatasi and P. sergenti (Fig. 2A, open and solid circles) developed lesions as rapidly and as large in size as the L. longipalpis SGL control group (Fig. 2A, open square) (P < 0.001), all being slightly larger than the group infected with parasites alone (Fig. 2A, solid square). Unexpectedly, a partial protection was also conferred by L. longipalpis SGL pre-immunization even when mice were subsequently challenged with parasites alone without L. longipalpis SGL (Fig. 2A, open triangle).

The differences among different groups in their lesion size are proportionally correlated with their parasite burdens estimated by limiting dilution method (Fig. 2B), i.e. L. amazonensis and L. longipalpis SGL co-inoculation (blank bar) > L. amazonensis alone (solid bar) >> L. longipalpis SGL pre-immunization followed by challenging infection + SGL (grey bar). Pre-immunization of mice with L. longipalpis SGL followed by challenging infection with or without SGL (grey and upward diagonal bars, respectively) decreases their parasite burdens to ~45% of those observed in the control group.
Cells were recovered from the ear dermis 2 weeks post-infection for flow cytometric analysis of their composition [4]. Used for such studies were five of the six groups (see Fig. 2), i.e.: (a) mice pre-immunized with L. longipalpis SGL followed by challenges with L. amazonensis promastigotes plus L. longipalpis SGL; (b) same as (a) without prior immunization with L. longipalpis SGL; (c) non-immunized mice inoculated with the parasites alone without SGL; (d) and (e) mice pre-immunized with non homologous SGL followed by challenges with L. amazonensis promastigotes plus L. longipalpis SGL. Mononuclear phagocytes (Fig. 3, circled lower gate) and neutrophils (Fig. 4, circled upper gate) were found ~1.5- to ~2-fold less abundant in the ear dermis of L. longipalpis SGL-immunized mouse group (a) (L. long + L. amaz/L. long) than those from the non-immunized groups (b) (L. amaz/L. long) and (c) (L. amaz) as well as those from the immunized groups with non homologous SGLs (d) and (e) (P. pap + L. amaz/L. long and P. ser + L. amaz/L. long, respectively). The difference between them in the number of mononuclear phagocytes was noted as early as day 2 after challenging infection [group (b) 19% versus group (a) 11.5%, data not shown]. Specificity of these cell populations identified is indicated by their absence in the control preparations (Figs. 3 and 4, lower panels). Also noted was a very small population of mononuclear cells, which were doubly positive for the F4/80 antigen expressed by murine macrophages (anti-F4/80 antibody) and MHC class II (M5/114 antibody) (Fig. 3, circled upper gate), suggestive of their identity as dendritic cells and/or activated macrophages known to have a high level of the latter on the surface [19]. In contrast to cells positive for M5/114 alone, these double-labeled cells were 2.5 to 4 times more in group (a) immunized with L. longipalpis SGL (L. long + L. amaz/L. long) than the non-immunized groups (b) and (c) (L. amaz/L. long and L. amaz, respectively) as well as those immunized groups with non homologous SGLs (d) and (e). These data were consistently obtained from three separate experiments, strongly suggesting that L. longipalpis SGL-immunized mice produce a significantly altered inflammatory response.
4. Discussion

In endemic sites under natural conditions, it is expected that humans are exposed to female sand flies of different genera and species, of which the majority is likely to harbor no Leishmania at all and, when they do, they may be of different species [20, 21]. For example, L. chagasi and L. amazonensis transmitted by sand flies of the genus Lutzomyia co-exist and cause visceral and cutaneous leishmaniasis in South America, respectively [22, 23].

In this laboratory study, BALB/c mice were used to evaluate the presence of genus- and species-specific immunomodulatory molecules. The sand fly–Leishmania species combination used, although unnatural, has long been accepted for such studies [5, 24], since it is difficult to rear the natural vector (Lutzomyia flaviscutellata) for L. amazonensis.

We showed that anti-saliva antibodies produced by mice repeatedly bitten by three different species of sand flies from two different genera recognize largely specific SGL antigens. This is consistent with our previous finding that the saliva-
vary antigenicity is species-specific among *P. papatasii*, *P. perniciosus* and *P. haeplensis* [11]. These findings are consistent with previous observations in other insect vectors, such as mosquitoes whose salivary proteins have long been known to antigenically cross-react only among those from closely related species [25].

In keeping with previous reports on the *L. major*—*P. papatasii* model [2,4–6], we demonstrate here in a different sand fly—host combination that pre-immunization of BALB/c mice with *L. longipalpis* SGL confers on them a protective phenotype against infection by *L. amazonensis*, whereas parasite-saliva simultaneous co-injection exacerbates the ear lesion. The latter effect has been noted in multiple *Leishmania*-vector combinations [4,12,26,27] and is thus not unexpected. Both effects reported here in the ear dermis model are less dramatic than previously reported for the *P. papatasii* SGL—*L. major* combination, although the fly species used here is of a different geographic origin [9].

Reported for the first time here is the finding that SGL-mediated protective immunity is sand fly species (e.g., *L. longipalpis*)-specific, since pre-exposure of mice to SGL of the other two sand fly species studied was ineffective. It awaits further investigation to elucidate the molecular basis of this species specificity. Maxadilan may be a potential candidate for consideration, since it appears to exist only in *L. longipalpis*, and since it exacerbates the ear lesion when co-injected with different *Leishmania* species i.e. *L. chagasi*, *L. braziliensis* and *L. major* [2,26,27]. Moreover, immunization of mice with maxadilan protects them against *L. major* infection by eliciting a Th1 type response [2]. It is of interest to note that a positive correlation exists between anti-*L. longipalpis* saliva antibody titers and the development of anti-*L. chagasi* DTH response in human infection [7].

In the present study, the observed protective phenotype is significant, but limited in scope (Fig. 2). The mechanism of this partial protective immunity in SGL pre-immunized mice is unknown. The simplest interpretation of this is antibody-mediated neutralization of SGL antigens, such as leukocyte-chemotactic factors [28,29], thereby reducing the mobilization of mononuclear phagocytes to the site of infection. Indeed, mononuclear phagocytes are relatively few in the partially protected group as compared to the others at both time points assessed, i.e. as early as day 2 (not shown) and week 2 after challenging infection (Fig. 3). Rapid provision of abundant host cells to *Leishmania* at the site of infection ensures their successful intracellular parasitism [30]. This may be crucial for *Leishmania* to escape from cytolytic humoral factors. Co-inoculation of SGL may conceivably enhance the provision of these factors to augment the early recruitment of monocytes in these groups of mice, contributing to the saliva-mediated increase of ear thickness, concomitant with a slight increase in parasite loads at week 16. Pre-immunization of mice with SGL may have additional effects on parasite survival, as it also lowers, at early time points after parasite inoculation (week 2), the number of neutrophils at the challenging sites (Fig. 4). *Leishmania* are killed rapidly after phagocyto-

dis by polymorphonuclear leucocytes (PMN) [31], which is a major component of the mammalian innate immunity to *L. major* infection. An increase in number of PMN and their persistence has been reported to sustain Th2 response in BALB/c mice [32], accounting in part for their susceptibility to *L. major* infection. Recent experimental data also suggest that *L. major* may survive in PMN, potentially facilitating their subsequent parasitization of macrophages; in addition, infected PMN secrete chemokines, which attract macrophages, the definitive *Leishmania* host cells [33,34]. It has been further reported that depletion of neutrophils results in a reduction of *L. major* infection in susceptible BALB/c mice [32,35], but exacerbates this in resistant mice [35]. Our results suggest a similar role of PMN in the *L. amazonensis*-BALB/c mouse model. In concordance with our observations, neutrophils have also been reported to persist in the lesions of BALB/c mice infected with *L. braziliensis* together with SGL [26]. Of particular interest is the preliminary evidence for the emergence of what appear to be antigen-presenting cells and activated macrophages with surface-expressing MHC class II molecules in SGL-pre-immunized mice (Fig. 3, circled upper gate). Whether this signifies an activation of effective T-cell immune response awaits further investigation.

Of interest is the protective phenotype observed in mice pre-immunized with *L. longipalpis* SGL and subsequently challenged with parasites alone without SGL (Fig. 2). This finding does not necessarily negate the potential functional importance of humoral immunity in human infection, since these parasites are naturally transmitted by sand fly vectors and thus always co-inoculated with salivary antigens. However, it is intriguing that their exclusion from the challenging inoculum still elicits an anti-*Leishmania* phenotype in SGL-immunized mice. This necessitates the consideration of additional or alternative non-specific mechanisms(s), i.e. innate immune responses via activation of gamma/delta T cells in the skin [36] or specific ones, i.e. parasite-saliva antigenic sharing in their glycan moiety [3]. There is currently no experimental data to support these assumptions. Whatever the mechanisms may be to account for the partial protective immunity observed, it is apparently insufficient to clear the parasites at least as observed in the model under investigation (see Fig. 2). This partial protection may, however, contribute directly or indirectly in part to the well-known fact that local inhabitants of the endemic areas are more resistant or tolerant than newcomers to leishmaniasis. Our observations are of importance in considering epidemiology and immunoprophylaxis strategy, since individuals in endemic areas are predictably bitten predominantly by uninfected sand flies of different vector and non-vector species, and since SGL molecules are under consideration as components of anti-*Leishmania* vaccine design.

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References


