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Research paper

Exploring new immunological insight on SP15 (~14 kDa) family protein in saliva of Indian sand-fly (*Phlebotomus argentipes*) in experimental visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is a disease caused by protozoan species of the genus *Leishmania* and is transmitted through bites from the *Phlebotomus* sand fly; it is associated with considerable morbidity and mortality in many parts of world, including India. Reports on the protective role played by saliva proteins of *Lutozomyia longipalpis, Phlebotomus papatasi and Phlebotomus duboscqi*. are available. However, no studies have explored the salivary proteins of *P. argentipes*, which is the known proven vector for the transmission of VL in the Indian sub-continent. Herein we revealed the presence of two proteins of 14.2 and one protein of 13.6 kDa in Indian strain *P. argentipes* which is absolute identical to previously reported protein of SP15 family (PagSP01, PagSP02 and PagSP07) of *P. argentipes* of NIH colony, USA. In an experimental study on *P. argentipes* from Bihar, India, we demonstrated that a strong humoral and cellular immune response was triggered to reduce the concomitant *Leishmania* load in groups of immunized mice. The immunized group produced a considerable amount of IgG antibodies, and their splenocytes generated TH1 cytokines (IL-12, IFN-γ) with the support of delayed-type hypersensitivity (DTH) reactivity in such mice at the challenged site. We summarize from our data that some identical proteins to previous from SP15 family protein of 14.2 and 13.6 kDa molecular size, derived from Indian *P. argentipes* and reported its first time, can also be significant in resolution of VL infection after modulation of host protective T cell response in VL.

1. Introduction

Leishmaniasis is a vector-borne disease transmitted by different species of infected female sand flies. It presents variable clinical manifestations from visceral to cutaneous and mucocutaneous. In the Indian sub-continent, visceral leishmaniasis (VL) is a serious health problem, and it is caused by *Leishmania donovani* and transmitted through the sand fly vector *Phlebotomus argentipes* in affected hosts; in contrast, in other parts of world, such as Sudan and Brazil, the vectors of the disease are *Lutozomyia longipalpis, Phlebotomus papatasi and Phlebotomus duboscqi*. Leishmaniasis as a whole is endemic in ninety-eight countries, with an estimated 350 million people at risk, and there are two million new cases each year [1]. According to a previous report, unfortunately, > 90% of the global visceral leishmaniasis cases are in India, Bangladesh, Nepal, Sudan and Brazil [2], and there are an estimated 60,000 deaths per year worldwide [3]. *P. argentipes*, the main culprit for the transmission of VL infection, may also be responsible for the transmission of the disease by biting PKDL cases, which appear to be a reservoir host for visceral leishmaniasis in India. The bioactive molecules present in the saliva of infected sand flies play a pivotal role in the establishment of natural infection, as the vector needs only a limited number (hundreds to thousands) of metacyclic promastigotes, whereas the laboratory infection requires millions of parasites alone to establish the infection [4]. However, a low number of laboratory parasites pulsed with the lysates of the salivary glands successfully establishes the infection in experimental mice [5]. Comparatively, the literature scarcely shows the relevance of bioactive saliva in the context of host protection, which contains as many as nine salivary proteins that can be immunogenic, and we reported the generation of antibodies against these nine salivary proteins and delayed-type hypersensitivity (DTH) after inducing the saliva proteins in the host through biting [6–8]. It is hypothesized that many of these saliva proteins render resting mononuclear cells active and may participate in protection in humans [9] by boosting the cellular arms of the protective immune

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response [7]. We previously showed concrete evidence for a role of the anti-saliva antibodies of *P. argentipes* in immune function and protection against VL [8]. Reports by other groups on the saliva of *Lu. longipalpis* are available, in which the LJM11 salivary protein was found as protective immunity against *Leishmania major* infection [10]. This study discussed a mechanism through triggering of DTH as well as an enhanced IFN-γ effect against Lu. *longipalpis* saliva protein in experimental mice [11]. The vector saliva protein of *P. papatasi* is also successful at protecting against *L. major* infection, and the protein is recognized as an SP15 family protein of a smaller size [12]. Several works also reveal the participation of vector saliva proteins of SP15 of *P. papatasi* [13] and *P. duboscqi* [14]. However, until now, no work has addressed the SP15 family proteins of *P. argentipes* collected from the Indian sub-continent [15], which has transmitted the disease visceral leishmaniasis for almost a century and requires a strong mode of disease protection and elimination. If the strong protection is availed by the SP15 (~14 kDa) family proteins of *P. argentipes* saliva, which are almost functionally similar to *Lu. longipalpis* and *P. papatasi* for VL and CL, respectively, then the SP15 family proteins might be a strong and unbeatable universal vaccine candidate for the prophylaxis of leishmaniasis.

Therefore, in view of several reports on the low molecular weight of *P. papatasi* and *Lu. longipalpis* saliva, the authors endeavored to study the SP15 (~14 kDa) family proteins of *P. argentipes,* which lacks a series of host protection, by using sand fly saliva to develop a strong vaccine candidate with no mammalian homologues [14]. In this study, the appearance of DTH and the development of antibodies, along with protective cytokines, were estimated in the immunized host by the induction of the eluted small-molecular-weight *P. argentipes* saliva proteins of the SP15 (~14 kDa) family to explore and report the immunity and the protection in an experimental model of visceral leishmaniasis.

2. Material and methods

2.1. Animal ethical clearance

The animals used in this study were approved by the Institutional animal Ethics committee (IAEC) of the ICMR-Rajendra Memorial Research Institute of Medical Sciences, Patna, India and guided by CPCSEA, Chennai, India before starting the work on the experimental animals.

2.2. Sand flies and SGH preparation

The uninfected sand flies of the species *P. argentipes* were collected from an endemic area of visceral leishmaniasis and were reared in the insectarium of the ICMR, Rajendra Memorial Research Institute of Medical Sciences, Patna, India. Adult female sand flies were offered a cotton swab containing 20% sucrose solution, and the salivary glands were dissected from 4 to 7-day-old female flies. The samples were stored in PBS (pH 7.2) at −80 °C until use. Before use, the glands were disrupted by the freeze-thaw method (3 cycles) and centrifuged at 12,000*g* for 2 min. The supernatant was collected and used immediately as a salivary gland homogenate (SGH).

2.3. Elution of the SP15 (~14 kDa) family proteins from P. argentipes saliva

For the elution of the Sp15 family proteins, SGH was subjected to SDS-PAGE using 13.5% resolution under reducing conditions. Gel slices containing ~14 kDa antigenic proteins were placed into separate micro centrifuge tubes, grinded and incubated in extraction buffer. The Gel slurry was transferred to a Nanosep MF device for the elution of the desired antigenic fragment. The sample (500 µl) of the protein solutions (100 µg/ml) containing the ~14 kDa fragment was centrifuged at 5000*g* in the Nanosep device to a final volume of 50 µl. The eluted proteins were again quantified by Lowry's method and checked by SDS-PAGE.

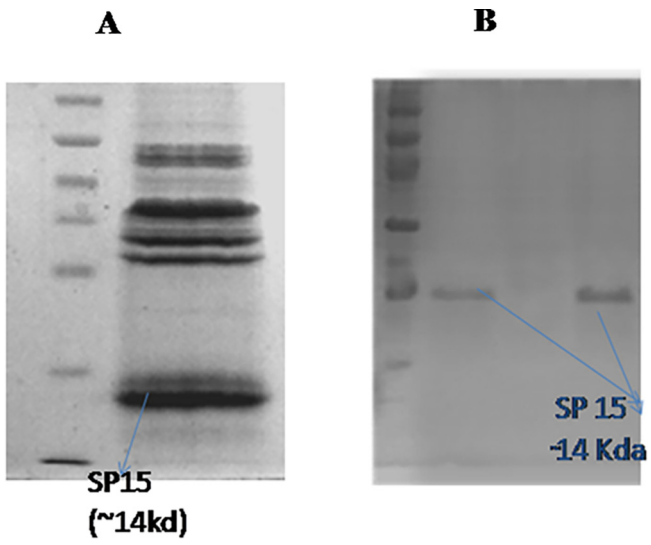

Fig. 1. (A) Segregation of the vector saliva proteins. (B) Elution of the SP15 (~14 kDa) family proteins of *P. argentipes.*

2.4. Mice

The procured 5 to 8-week-old, 18–20 g inbred BALB/c mice were purched from the Central Drug Research Institute, Lucknow, India and were maintained in the Animal House of the ICMR-RMRIMS under hygienic conditions, and all the mice were given a balanced diet with water *ad-libitum*.

2.5. Conformation of the DTH response by ear thickness and ear dermis inflammatory cells

For DTH confirmation, the mice were intra-dermally immunized three times at two-week intervals with 500 ng of eluted SP15 family (~14 kDa) proteins from *P. argentipes* in a volume of 10 µl each time in the left ear. Two weeks after the last injection, the SGH was challenged in the right ear, and the DTH response was observed by measuring the ear thickness at 72 h using a Vernier caliper from the dorsal to the ventral side of the ear. For the inflammatory response analysis in the ear dermis, the mononuclear cell infiltration, composed mainly of macrophages and lymphocytes, was assayed in the immunized mice at 72 h of exposure to SGH in the right ear [10]. For this, the ear of the culling mice was rinsed in 70% ethanol and dried. A skin snip of the ear was taken for smear preparation. The smear was fixed with alcohol and stained with Giemsa stain for a microscopic examination of the mononuclear cell infiltration causing the inflammatory response.

2.6. Antibody detection by western blotting

For the detection of the antibodies, SGH and the eluted proteins of the SP15 family (~14 kDa) were subjected to 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein procured by SDS-PAGE was transferred to a nitrocellulose membrane using transfer buffer (20 mM Tris, 150 mM Glycine, 20% methanol) on a Blot-Module for the xCell-II mini-cell followed by blocking with blocking buffer (Tris pH 8.0 plus 150 mM NaCl plus 5%BSA) at 4 °C overnight. Furthermore, the membrane was washed with TBS-T and was incubated for 1 h with the sera (1:500 dilution) of the immunized and non-immunized mice. After one more wash, the membrane was incubated with secondary horseradish peroxidase (HRP) conjugated with anti-mice IgG (1:1000) antibody. Hydrogen peroxide (H2O2) and diaminobenzidine (DAB) were used to access the protein bands.