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Direct evidence for role of anti-saliva antibodies against salivary gland homogenate of *P. argentipes* in modulation of protective Th1-immune response against *Leishmania donovani*

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ABSTRACT

Currently the main concerns regarding control of visceral leishmaniasis (VL) caused by *L. donovani* are immunosuppression, relating toxicity of anti-leishmanial drug and little development in appropriate vaccine and vector (*P. argentipes*) control. Reports available from *ex-vivo* studies reflect significance of vector salivary gland homogenate (SGH) in reverting immunosuppression of infected VL subjects and as such the immunogenic nature of SGH can be a strategy to modulate immune system and anti-leishmanial function to enable immune response to control the disease. Several related studies also identified a better utility of vector anti-saliva antibodies in achieving such effects by an adoptive transfer approach instead of direct stimulation with SGH protein. However, conclusive evidences on VL cases are far beyond satisfactory to suggest role of SGH into modulation of host immune response in VL subjects in India. This study was under taken to make comparison on change in cytokines (TH_1 and TH_2) response pattern and anti-leishmanial macrophage (Mϕ) function following stimulation of their $PBMC_S$ with SGH protein derived from *P. argentipes* sand fly vector for VL or anti SGH antibodies raised in rabbit.

This study reports for the first time that *L. donovani* sensitized healthy subject demonstrates an up-regulated Interferon-γ (TH_1) and down regulate Interleukin-10 (TH_2) production following stimulation of their PBMCs by *P. argentipes* anti-saliva antibodies accompanied with an improvement in anti-leishmanial Mϕ function for nitric oxide (NO) production. Subsequent experiments suggest that *P. argentipes* based anti-SGH antibodies when used to stimulate LD infected PBMCs in healthy subjects resulted in better clearance of *Leishmania* amastigotes load compare to SGH protein. Possibly the immunogenic components of anti-saliva an antibody maintains the level of protective cytokine (INF-γ) and seems to restrict the infection by host protection by vector saliva.

1. Introduction

Visceral leishmaniasis (VL) in the Indian subcontinent, also known as kala-azar, caused by *Leishmania donovani*, is fatal if not treated and responsible for an estimated 60,000 deaths per year worldwide [1]. More than 90% of the world's reported VL cases are in India, Nepal, Bangladesh, Sudan, and Brazil [2]. In India, Nepal, and Bangladesh *L. donovani* is transmitted exclusively by bite of infected female sand fly, *Phlebotomus argentipes*, which causes anthroponotic transmission of VL [3]. Role of Sand fly saliva either in disease progression or protection is extensively studied by several authors as Immunomodulatory role of *L. longipalpis* and *P. papatasi* saliva and protective molecules such as Maxadilan of *L. longipalpis* and PpSP15 of *P. Papatasi* against leishmaniasis [4–6]. *L. longipalpis* saliva effects complement activation, haematopoiesis, T-cell proliferation and functions [7]. Pre-exposure to uninfected *P. papatasi* bite or pre-immunization with salivary gland homogenate (SGH) decreases/prevents the disease progression in host [8].

Host Protection against the disease may be due to the production of antibody against SGH and that may neutralize disease promoting molecules/components present in the saliva proteins of *P. papatasi* [9]. Repeated exposure of *P. papatasi* bites develops antibodies against vector saliva in experimental animals has been reported previously and antibodies are specific to the local sand fly species [9–11]. Anti-saliva antibodies formation has also been reported in humans however, intensity of recognition varied among individuals [12–14].

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The natural infection by the infected sand fly requires only a very small number (hundred to thousand) metacyclic promastigotes together with strong bioactive saliva to establish the infection whereas the laboratory infection needs million of parasites to establish the infection [15]. The saliva of *L. longipalpis, P. papatasi* inhibits the lymphocyte proliferation to suppress the early production of INF-γ [12] and down regulates the expression of inducible NO synthase (iNos) [16,17]. A series of reports has been published regarding the *Leutzomia longipalpis* and *Phlebotomus papatasi* saliva which reveals the protection/disease manifestation in the concerning area. But information on Indian *Phlebotomus argentipes* saliva where host protection may be confirmed by specific saliva antibodies of the vector is not available. Possibly the disease is endemic due to antibodies raised by uninfected fly in new area.

The saliva is a gamut of many proteins as *P. papatasi* saliva shows nine salivary proteins after characterization. The protein of 15 kDa (PpSP15) has protective role against *L. major* in mice model [6]. In *L. longipalpis* saliva, a novel protein of 11 kDa (LJM19) has protective role in hamster against VL and immunized hamster which maintains low parasitic load and inducible NO expression in spleen and liver [18]. We have also allocated the different protein bands in the saliva of *P. argentipes* and their functions are under the study. Presently the authors have extensively reported the role of *P. argentipes* saliva of Indian set up through the effect of SGH, Ld pulsed SGH and anti-saliva antibodies procured from experimental animals which are used routinely for sand fly feeding.

2. Materials and methods

2.1. Ethical and informed consent clearance

This study was approved by Ethical committee of Rajendra Memorial Research Institute of Medical Sciences (ICMR), Patna, India and informed consent was taken by each donor of the samples.

2.2. Sand flies and SGH preparation

The vector sand flies of genus *P. argentipes* (Indian strain) were reared in the insectariums of Rajendra Memorial Research Institute of Medical Sciences (ICMR), Patna, India. Adult female sand flies were offered a cotton swab containing 20% sucrose solution and 4–7 days old flies were used for dissection to collect the salivary glands in 20 μl of phosphate buffer saline (PBS, pH 7.2) on ice. The glands were disrupted by freeze & thaw method (3 cycles) and centrifuged at 10,000*g* for 2 min. The supernatant was collected and stored at −80 °C [19]. Protein was quantified by Lowry's method.

2.3. Parasite culture

The cryopreserved reference strain (MHOM/N/1983/Ag83) was recovered and maintained at 24 ± 1 °C in RPMI 1640 medium (Sigma, St Louis,MO) supplemented with 10% FBS (Hyclone, Logan, UT), 20 mM L-glutamine, 50 U penicillin, 50 μg streptomycin, 25 μg of genetomycin/ml. The infective stage metacyclic promastigotes of *L. donovani* culture were used during experiment.

2.4. Peripheral blood mononuclear cells isolation

Peripheral blood was collected in Na_2 heparin (BD) from healthy individuals and PBMCs were isolated over Histopaque1077 (Sigma-Aldrich, USA) by density gradient centrifugation (200*g* for 15 min). The obtained cells were washed with PBS and counted by using 0.1 mm Naubauer chamber (Fein Optia, JENA, germany).

2.5. Optimization of SGH proteins concentration for maximum activation of lymphocytes

Maximum activation of lymphocytes was measured with expression level of CD69. For this PBMCs (10^6/ml) were cultured with different concentration of SGH protein (30, 15, 7.5, 3.7, 1.8, 0.9, 0.4, 0.2, 0.1 0.056 μg/ml) for 24 h on 36 ± 1 °C in 5% CO_2 incubator with 95% humidity. The cells culture was transferred in FACS tube and washed with PBS. The cells were labeled with surface anti-CD69- FITC antibody (BD) for 30 min and were fixed with cytofix followed by stain buffer. Expression of anti-CD69 cells were acquired by FACS-Calibur Flow-Cytometer (Becton Dickinson, San Diego, USA) within 24 h [20] and the results were analyzed by using BD Cell Quest Pro Software (Becton Dickinson, San Diego, USA).

2.6. Fluorescence Activated Cell Sorter (FACS) based intracellular cytokine assay

The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). The cells (10^6/ml) were stimulated with optimized SGH (0.9 μg/ml) concentration, Ld ($0.05x10^6$/ml), SGH pulsed Ld and anti-saliva antibodies (10 μg/ml) to evaluate the expression level of IFN-γ and IL-10 on $CD4^+$ cells.

The cell culture condition involved humidified 5% CO_2 incubation at 35 ± 1 °C and 4 h before completion of incubation, Golgi stop (1 μl/ml) was added to stop the reaction. The culture was transferred in FACS tube and centrifuged @ 200*g* for 20 min on 4 °C. The obtained cells were washed with PBS and labeled with surface anti-CD4 antibody (BD) for 30 min in dark. The cells were fixed with cytofix followed by centrifugation with stain buffer and finally Perm wash solution was then added with labeled antibodies (PE IL-10 and FITC IFN-γ) and re-suspended in stain buffer. For detection of Cytokines (IL-10 and IFN-γ) on $CD4^+$ cells, the cells were acquired and analyzed through FACS-calibur Flow-cytometer (Becton Dickinson, San Diego, USA) within 24 h. The percentage of IFN-γ/IL-10 containing cells was analyzed by using BD Cell Quest Pro Software (Becton Dickinson, San Diego, USA).

2.7. ELISA based extracellular cytokines assay

ELISA was performed to measure the quantities of extracellular cytokines (IL-10 and IFN-γ) in pg/ml. For this, supernatants of another set of cells culture were used. Cytokine level was determined by using commercial anti-cytokine antibody as described by manufacturer (BD OptEIA). Absorbance was taken at 450 nm in micro titer plate reader (Bio Rad). Human recombinants IFN-γ and IL-10 (BD OptEIA) were used to generate standard curves. Samples were analyzed in triplicate.

2.8. Parasitic load determination

For parasitic load, mononuclear cells were separated from human PBMCs and monocytes derived macrophage cells (0.05×10^6/well) were cultured in 8 wells Lab tech chamber slide at 36 ± 1 °C at 95% humidified in 5% CO_2 maintained incubator. Non adherent cells were removed and adherent cells were cultured in complete medium for overnight. The cells were challenged with required number of Ld, SGH pulsed Ld. Anti-saliva antibodies prone cells were also challenged with SGH pulsed Ld for 24 h to establish the infection. Cells challenged with Ld were kept as control [21]. The establishment of infection was examined after completion of 24 h incubation.