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Transmembrane Immunization with Leishmania exo-antigens free of Adjuvants.

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Abstract

We have examined the immunological response to *Leishmania donovani* promastigote derived exo- antigen in a mouse model without any adjuvant. The topical application of *Leishmania* exo-antigen to the intact skin, nasal, or buccal mucous membranes of naïve BALB/c mice elicited antibody and cytokine production to levels that were detectable in plasma for up to five or six weeks post sensitization. Remarkably, these antigen specific immune responses observed did not require co-administration of adjuvant and were detectable after only one or two applications of 50 to 80µg of product. The most effective sensitization route for antibody production was through contact with the buccal mucosa when significant levels of IgG1 and IgG2a subtypes were measured. More importantly, a single injection of 100µg of exo-antigen subcutaneously in mice elicited the protective helper T-cell (Th-1) phenotype manifested by high levels of IFN-g (8 to 10x) and no IL-4 production. This highly desirable profile was not observed with experimental infections or when exposure to exo-antigens took place through contact with the skin, nasal, or buccal mucosa. This finding of a novel parasite antigen which selectively induces the protective Th-1 phenotype when administered subcutaneously into a murine host without the co-administration of an adjuvant is unusual. Here is a way to induce protective immune response against *Leishmania* Parasites with *L. donovani* Exo-antigen. This approach is promising for future experiments/immunizations in humans, on the development of potent vaccination against leishmaniasis and possibly other infectious organisms.

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Introduction

Although hamsters and guinea pigs were used as animal models earlier on, the inbred strains of mice with defined genetic background were later on found to be more reliable to investigate the immunological responses against *Leishmania* parasites. *L. tropica*, *L. major* and *L. donovani* infections in mice have been extensively used as a model for human leishmaniasis. Subsequent publications showed a spectrum of resistance and susceptibility in mouse models. At least three loci H-2, H-11 and Ir-2 are known to regulate the course of *L. donovani* infection in mice. Extremes are represented by BALB/c and their H-2 congenics (susceptible to chronic cutaneous disease, visceralization and premature death) and CBA/H resistant in that cutaneous disease is minimal and of short duration) and C3H/He and C57Bl/6 being relatively resistant. With the *L. major* infection, BALB/c mice are known to be very susceptible and even as low as 20 promastigotes, a progressive local lesion seen followed by visceralization and fatal outcome. The C3H/He and C57/Bl are known to be fully resistant. The susceptibility to *L. major* is regulated by genes H-11. This model is useful for characterization of host immunological responses. Inbred mice have been extensively used as model for leishmaniasis. In recent years, genetically mutant and gene knock-out mice offer several possibilities to perform experiment and provide immunological explanations and specific indications. Nothing is known as yet of the formal human genetic basis of susceptibility to leishmaniasis. Marginally some information exists on cutaneous disease but only as anecdotal level. A range of clinical manifestations seen in cutaneous type is due to host rather than parasite variation.

Immunization across intact membranes has been described in the past. Glenn et al. (1998a, b) induced mice to mount an immune response to bovine serum albumin trans-cutaneously by the co-administration of cholera toxin (CT). This observation has been extended to other antigens to include vaccines already in common use globally. Importance of immunomodulatory oligonucleotides (GpG) have been described (Ho et al., 2018). More recently, transmission blocking activity was demonstrated in mice immunized by the intranasal application of *P. vivax* Pvs25 combined with cholera toxin. The cholera toxin plays the role of adjuvant in these experiments. The previously held belief that the adjuvanting properties of CT and LT were related to ADP-ribosylation has been put in doubt by the finding that other molecules such as GpG oligos (Ho et al., 2018) with no ADP ribosylation

activity exhibit similar adjuvant activity. Despite the fact that the precise molecular mechanism of action is unknown, the adjuvant essentially stimulates resident Antigen Processing Cells (APCs) to engulf and process the co-administered antigen. The activated APC then migrates and subsequently presents the processed antigen to the appropriate MHC system. Antigen presentation frequently takes place away from the site of antigen application in a draining lymph node or spleen. In this manner, a specific immune response is generated to the adjuvant and the sensitization agent (Pasquale et al., 2015).

Another critical role played by co-administered adjuvant is to potentially drive the immune response of the host away from the susceptible Th-2 towards the protective Th-1 phenotype. The paradigm for the helper T-cell dichotomy and its relationship to disease was first elucidated in *L. major* infection in the mouse model. In the resistant mouse, infection with *L. major* leads to IL-12 associated priming of CD4+ T-cells with IFN-gamma production and stimulation of macrophages (mϕ) to destroy their resident intracellular parasites. In contrast, the susceptible BALB/c mice preferentially produce IL-4 in place of IFN-gamma. It is the production of IL-4 rather than the absence of IFN-gamma that interacts with IL-10 and probably IL-13 to confer susceptibility, disease and ultimately death, if left untreated. In BALB/c mice, there is a possibility that effector cells are rendered impotent by the reduced expression of H-2 coded associative recognition structures on infected mϕ, rendering this mouse strain highly suitable for the study the immune response. Evidence exists that expression of MHC antigens including Ia antigens in *Leishmania*-infected BALB/c macrophages is defective (Handman et al., 1979). Such a defect may facilitate induction of suppressor T-cell -dependent responses. Plasma IgG response is found to be under the control of I-A region of the H-2 major histocompatibility complex (Elson & Edding, 1985). Hence, inbred mice with H-2b (C57BL/6) or H-2q haplotypes - were high responders strains with the H-2s and H-2k (C3H) low responders and those with the H-2d (BALB/c) haplotypes were intermediate responders.

The central role that adjuvants play in vaccine delivery systems is now well-established and this has intensified the search and approval of safe and effective compounds for use in humans (Petrovsky & Aguilar, 2004). However, only a few compounds are approved for human use, impeding rapid progress in the timelines for taking several vaccine constructs for leishmaniasis and other diseases into clinical trials.

The route of administration of antigens has also been shown to influence the immune response mounted by the host. This variation in response and effectiveness may stem from the resident APC's at the site of application or the APCs that are selectively stimulated to take up the foreign molecules. In this regard, subcutaneous injection of antigen has been shown to be a preferred mode of antigen administration in cases where a predominantly Th-1 response is intended because of the ubiquitous presence of Langerhan APCs in the dermis of the mammalian host.

We have demonstrated that BALB/c mice can be induced to mount a robust antibody and cytokine response to a novel *Leishmania* exo-antigen produced in a protein-free medium. Sensitization takes place with application on to unbroken skin or mucous membrane without use of an adjuvant. The specific profile of the immune response varies with the mode of antigen delivery. Subcutaneous injection of 100 µg of exo-antigen engenders a Th-1 cytokine profile with the preferential production of IFN-gamma over IL-4.

Materials and Methods

In vitro propagation of *Leishmania donovani* promastigotes

Promastigotes of *L. donovani* WR 130E strain were obtained from the Walter Reed Army Institute of Research archive and cultured in vitro using Schneider's Drosophila medium (GIBCO-BRL) supplemented with 10% FCS (GIBCO-BRL)). Initially promastigotes were cultured in 75 cm2 flasks (Costar) expanded and pooled until sufficient quantities allowed transfer into roller bottles for large scale production of promastigotes.

Promastigotes for antigen production

The parasites in the expanded roller bottle cultures were transferred into 50 ml tubes and centrifuged at 2500 rpm for 15 min. The resultant supernatant was removed and the parasite-pellet reconstituted to the original volume with ice-cold XOM™ medium (protein-free culture medium-GIBCO-BRL). After seventh wash the promastigotes were transferred into a fresh roller bottle, 250 ml of sterile and warm XOM medium was added and incubated at 26 ±2 C for 5-7 days. A 10 ml aliquot was placed into a 75 cm^2 flasks to monitor the condition of promastigotes in the antigen cultures. Thereafter, the culture was centrifuged in 50 ml tubes at 3000 rpm x 4 times The final supernatant was filtered through 0.2 um pore-size Millex-GV filters (minimum protein binding durapore filters) bottled and stored at 4C until use. An aliquot of each batch of *Leishmania* exo-antigen preparation was used for protein determination. The promastigote antigens were used for immunization of mice and (b) for coating the wells for ELISA and cytokine assays.

Experimental animals:

Genetically inbred strains of mice, purchased from the Jackson Laboratories, were maintained under strict protocol approved by the Animal Use Ethical Committee of the Walter-Reed Army Institute of Research. A total of 33 BALB/c mice, aged 6 weeks were split into 11 groups comprising 3 mice each. Mice were tagged on the right ear for identification purposes and offered food and water ad libitum.

Animal restraining.

A cocktail of ketamine (110 mg/kg dosage) and xylazine (11mg/kg dosage) was injected intramuscularly to anesthesize the mice for sensitization with *Leishmania* exo-antigen. When animals reached full induction of anaesthesia, they were sensitized with *L. donovani* exo-antigen. For sample collection (saliva, blood, faecal pellets and urine), mice were restrained by hand.

Experimental groups and sensitization of mice with antigens by different routes.

The mice were divided into11 groups (n=3) three groups for each route (skin, buccal, and nasal) with three different doses of antigen (20, 100 and 500 µg in 25 µl volume) tested by each route. One group of mice (n=3) received 100 µg antigen by the sub-cutaneous route and the last group (n=3) served as controls. When animals reached full induction of anaesthesia, they were sensitized by application of antigen directly onto the pinna on the ears (skin group) precleaned with a cotton swab wetted with 70% isopropyl alcohol and the anaesthesia was extended until the applied antigen completely dried up. Antigen was instilled into the nares (nasal group) and applied to the oral mucous membrane (buccal group) with the help of sterile Pasteur pipettes. Antigen was also injected (injected group) subcutaneously with the help of sterile syringe and needle. The sensitization with antigens was repeated after 2 weeks into all sites. The control group did not receive any antigen.

Specimen collection

Microfuge tubes were appropriately pre-labelled with the animal tag # and the Whatman filter papers pre-smeared with the salt solutions, punched out into small discs collected into a petri-dish prior to sample collection. Required material for specimen sampling were carried over to the animal house. Animals were warmed-up for 5-10 min under a Pyrex flood light as a source of heat and picked up from its cage to collect a urine sample. The mouse was then hand-restrained for collection of the saliva sample onto the Whatman paper disc. Thereafter, the mouse was housed in a restrain-chamber, its tail pulled out and partially incised with a sharp blade. The blood sample

could accumulate on the incision, aspirated with a micro-pipette, and transferred into a microfuge tube. The restrained animal frequently defecated during blood collection and a few faecal pellets were taken. In instances where no faecal pellets were expelled, placement in the restraining chamber was repeated until successful.

Collection and processing of individual samples

Blood: With appropriate animal label were arranged in a rack according to the animal group/experiment. These tubes were used for collection of blood sample. Between 40µl and a 100µl blood was routinely collected from each animal and placed in a microfuge tube spotted with 20µl anti-coagulant solution. Blood samples were collected weekly and processed immediately. The plasma sample was separated by centrifuging in a microfuge at 12,000 rpm for 10 min. The resultant plasma sample was placed in appropriately labelled corresponding tubes and frozen at –20°C until used.

Saliva: Saliva samples were collected by using pre-coated salt discs. Whatman filter paper #1 was coated with salt solution by immersion in 0.85% NaCl solution for 10 min and air dried at 37 C. 0.5 cm punched discs were prepared and used for collecting the salivary specimens. Two to three discs were inserted into the mouth of the mouse using a pair of fine forceps and allowed to fully absorb saliva. They were then transferred into a microfuge tube pre-loaded with 100 µl of mammalian tonicity buffer supplemented with protease-inhibitor, PMSF (0.01M concentration). The labelled microfuge tubes with the filter paper discs were frozen at -200 C until used. On the day of testing, the tubes were thawed at RT and centrifuged at 12,000 rpm in a microfuge. The supernatant was transferred into fresh tubes and into the microtiter wells for IgA determinations. The neat sample in microtitre wells were frozen at -20° C.

Urine: The urine specimens were collected into microfuge tubes and frozen at –20° C until used.

Enzyme-linked immunosorbent assay

Preparation of test plates

Leishmania donovani and *L. major* exo-antigens were used at concentrations of 50 and 80 µg/ml respectively diluted in coating imidazole {0.017 M imidazole (ICN) 1.16g/litre supplemented with 0.15 m NaCl pH 7.3} buffer. The same batch of Leishmania exo-antigen used for sensitizing the mice was also used for coating of the ELISA plates. 75 µl of antigen solution was transferred into each well, the plate sealed and left overnight (16-18 h) for immobilization at RT. Antigen coated wells were blocked with 0.5% casein solution. Vials of casein kept frozen in -20 C freezer were thawed, mixed, and brought to ambient RT by immersing in the hot water. The unbound antigen solution in the wells was discarded and wells were blocked (250 µl/ well) with blocking solution for 45 min at RT. Plates were used for antibody measurement.

Total IgG, IgG1, IgG2a, IgM and IgA ELISA

Testing plasma, saliva and faecal elutions.

Plasma samples in duplicate wells tested at 1/200 dilutions, Saliva and faecal elutions at 1/25 dilutions diluted in PBS/T. All analyte samples were incubated for 1 h at RT. After analyte incubation, wells were washed with PBS/T and respective conjugate (HRP labelled anti-mouse total IgG, IgG1 and IgG2a, IgM and IgA conjugates) were added to wells and further incubated 30 min at RT. Thereafter, plates were washed 5x with PBS/T and allowed to dry. Freshly prepared TMB was added and the substrate allowed to develop colour for 30 min. A stop solution (1M H3 PO4) was added and the plates were read at 450 nm and absorbances were recorded.

Cytokine ELISA assays.

The cytokine (IL-4 and IFN-g) ELISA tests were optimised using commercially available reagents (Endogen and Cedarlane). Wells were coated with 2 ug/ml of capture PAb (goat anti-mouse IL-4 PAb or goat anti-IFN- γ PAb) in imidazole buffer and immobilized by incubating the plate O/N at RT. The following day, after removal of the unbound solution wells were blocked with 0.5% casein for 45 min. Neat Mφ culture supernatant or freshly thawed plasma diluted to 1/25 in PBS/T were added to the blocked wells and incubated for 16 h at RT. The following day the biotinylated anti-IL-4 or IFN-g antibody was added to the wells without washing and plates were incubated for an additional 1 h at RT. The conjugate streptavidin-HRP diluted to 1/4000 in PBS/T was added to each well and incubated for 30 min at RT. With another series of washing, TMB substrate was added as a substrate and allowed to develop the colour for 30 min. Plates were read at 450 nm after stoping the reaction with stop solution. Cytokine values were read off an optimized standard curve.

Results

Visualization of Leishmania parasites

Leishmania major parasites were directly stained using exo-antigen based polyclonal antibody labelled with FITC (Fig 1).

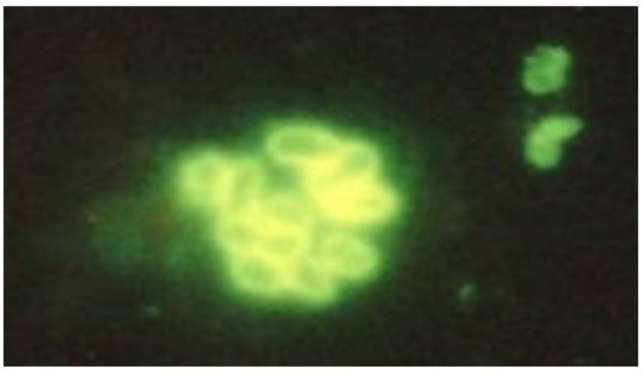

Fig 1: Direct staining of *leishmania* parasites using Exoantigens based polyclonal antibodies labelled with FITC

Impression smear was prepared from control infected animals and stained (Fig 2).

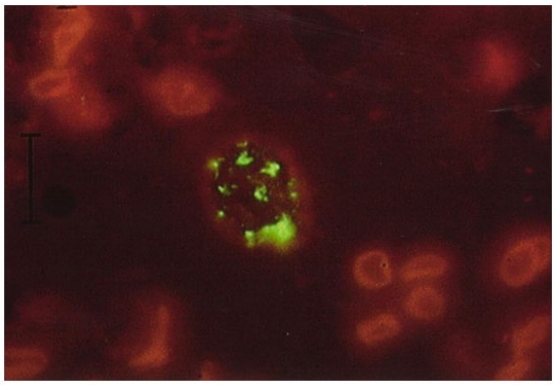

Fig 2: Impression smear from the foot lesion of *L major* infectedBALB/c mouse and the smear was stained by direct IFAT using Exo-antigen based polyclonal antibodies.

No such parasites were demonstrated in antigen sensitized animals.

Optimization of Immune response in infected animals and its comparison with the age-matched and naïve controls.

The immune response was studied in BALB/c mice infected with *L. major* and its variation if any with the age of mice was monitored in age-matched and naïve mice. Mice were infected during 5-Wk of their age and age-matched controls were kept. Both infected as well as age-matched controls were bled when infection is 5 months along with naïve 5-wk old mice control mice. Using *L. major* promastigote exo-antigen, total IgG, IgG1 and IgG2a was monitored. Fig 3 shows the kinetics of the response when promastigote exo-antigens were titrated.

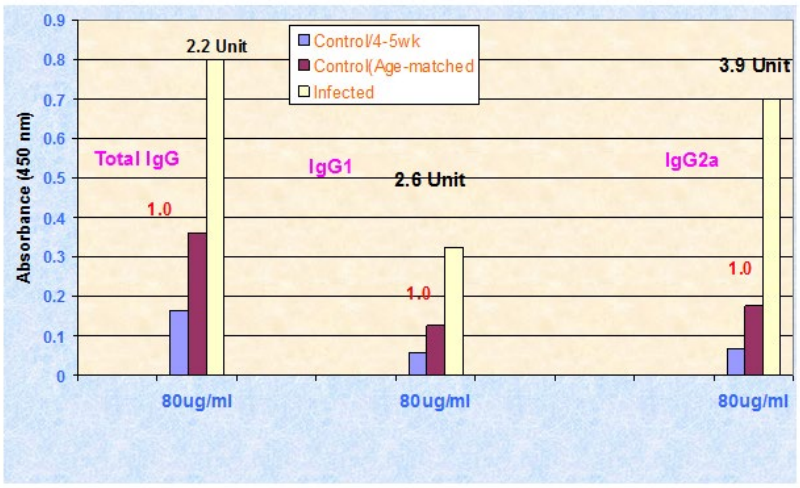

Fig 3: Total IgG, IgG1 and IgG2a level seen in Control, Age-matched and Infected animals

A distinct difference was apparent between age-matched and naïve controls. This data has shown the importance of matching the controls with appropriate age. In the next step, using the resultant absorbance at 80 µg/ml antigen concentration, the data is converted into the units higher than the age-matched controls (Fig 3). With this, variations occurring is reduced keeping the activity of control as the basal unit.

IgM response due to antigen sensitization

It was attempted to demonstrate the antibody response generated after sensitization with Ld-ESM by different routes and subsequent challenge with *L. major* . The IgM response in groups of mice sensitized with *L. donovani* promastigote exoantigens through skin, nasal, buccal routes and when injected through subcutaneous route (SC-Inj). Based on the level seen in age-matched control animals as the basal unit, a positive response was seen in skin and buccal route. The response was dose-dependent in the buccal exposure during Wk-3 and Wk-5. There was no significant increase in nasal or SC-Inj routes.

Total IgG and antibody subtype response

Determination of total IgG in ELISA clearly establishes the fact how an active and dynamic immune system elicited following the sensitized with the promastigote antigen as the non-self in absence of any adjuvant preparation. To minimise the variations within the assay, all three types of antibody determinations (ie, total IgG, IgG1 and IgG2a) were performed on the same plate and in the same assay. In other words, each diluted plasma sample was assayed for all three types of antibody indicator. Total IgG response is an indicative of humoral response in general whereas IgG1, an indicative of Th-2 response and IgG2a is that of Th-1 response. Fig 4 shows the total IgG response against sensitization with 20 µg (Skin 1, Nasal 1, Buccal 1), 100 µg (Skin 2, Nasal 2, Buccal 2) and 500 µg (Skin 3, Nasal 3 Buccal 3) in 25 µl volume.

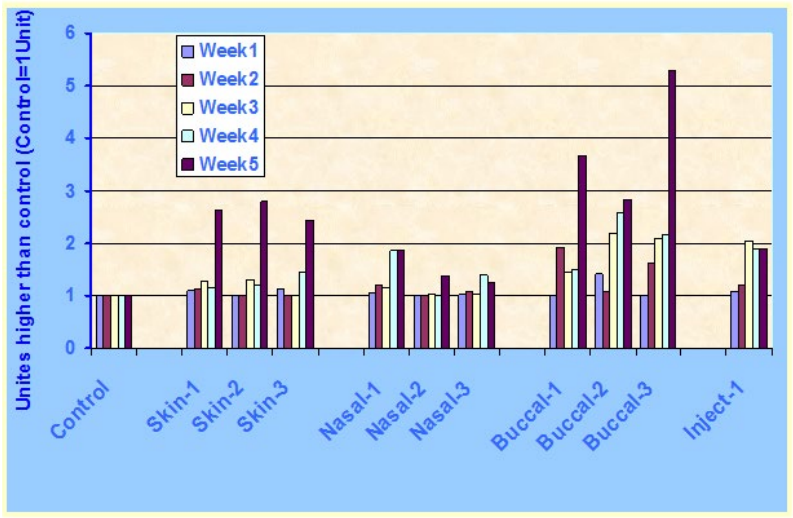

Fig 4: Total IgG response after sensitization

A positive response was evident in buccal and skin routes. The response was 3-4x higher in buccal route during Wk-5 where as in the skin the response was 1.5x that of controls. Sensitization via buccal response has generated a very predominant response which peaked during Wk-4 and Wk-5. Some difference was clearly apparent with the antigen dose. Peak levels were 3 to 4x higher than the controls. Once again, the SC-Inj and nasal routes have generated much subdued response.

IgG1 response

IgG1 response is the hall mark of Th-2 cellular response and characteristic to the non-healer response as well as the response suggests that antigen stimulation initiate Th-2 CMI response. Fig 5 shows buccal route has generated a spectacular IgG1 response which is significantly higher response than any other route.

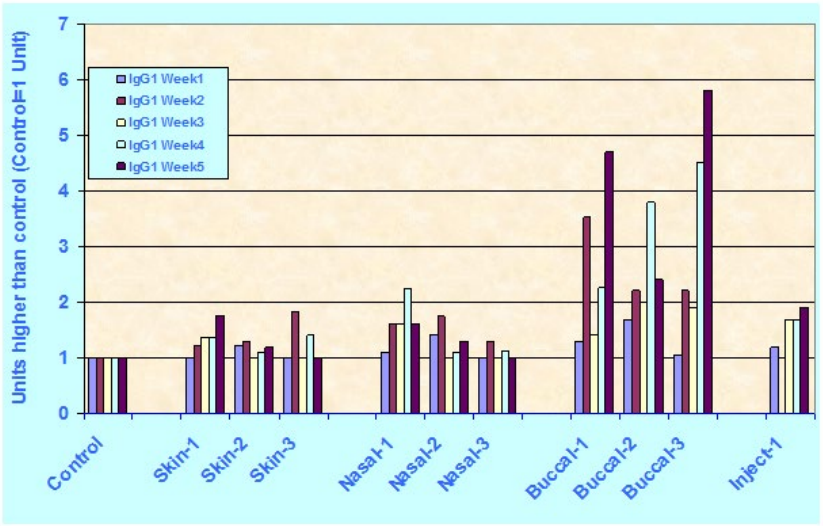

Fig 5: Total IgG1 response after sensitization

There was an indication of IgG1 response in the nasal route and SC-Inj. Antigen dose as low as 25 µg, in the skin route, has elicited highest antibody response than the higher dose. The mechanism regulating the IgG1 secretion seems to be dependent upon the Th-2 response and also antigen processing by the dendritic cells residing in the buccal route.

IgG2a response

IgG2a response is the hall mark of Th-1 cellular response. Fig 6 shows the IgG2a response when exo-antigen was used as the sensitizing antigen.

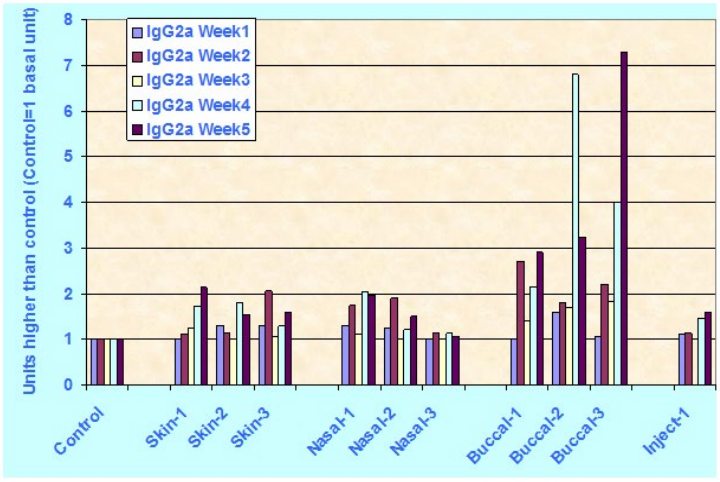

Fig 6: IgG2a response in sensitized animals

Once again, a spectacular response was evident in the buccal route and the response was 2 to 6x higher than the age-matched controls. The next route to show appreciable response was skin, nasal and SC routes. Once again the response was clearly evident during Wk-4 and Wk-5. The response was not associated with the antigen dose. It clearly indicated that leishmanial exo antigen has the ability to induce a protective response in absence of any adjuvants. The response by skin route was consistent and to some extent dose related. The IgG2a response in general is the response associated with the buccal route and skin route.

Salivary IgA response

Using saliva as the target specimen, the IgA response was monitored in BALB/c mice sensitized with *L. donovani* Exo-antigen through different exposure routes. The nasal, buccal and SC routes induced high levels of IgA (data not shown). Comparatively a relatively high response was observed in nasally sensitized animals and then to some extent in the buccal group. Response seems to be almost immediate through nasal route and maintained higher than controls throughout. Response was not dose-related. In general, some animals have showed nearly 2-3x higher response compared to controls. Highest response was evident through SC-Inj route.

Cytokine Assay Results

Optimisation of IL-4 and IFN-y assays are important to monitor the response in sensitized animals. Using commercially available reagents, IL-4 and IFN-yassays were optimised and the sensitivity level was checked with reference to the standard graphs constructed by using the reagents from 1 pg to 1000 pg level. The IL-4 assay was found to be sensitive 10 pg onwards but lacked sensitivity any value lower than 10 pg. The standard graph was steep and highly linear from 10 pg onwards resulting with very high R2 value. The standard graph of IFN-γ on the other hand, showed very high sensitivity from 1 pg/ml concentration. The graph was almost linear through out with very high R2 value. Based on this graph, the ELISA assays performed were highly sensitive from 1 pg/ml onwards.

Cytokine response during antigen sensitization

IL-4 response

Fig 7 shows IL-4 response with relative units higher than that of age-matched controls.

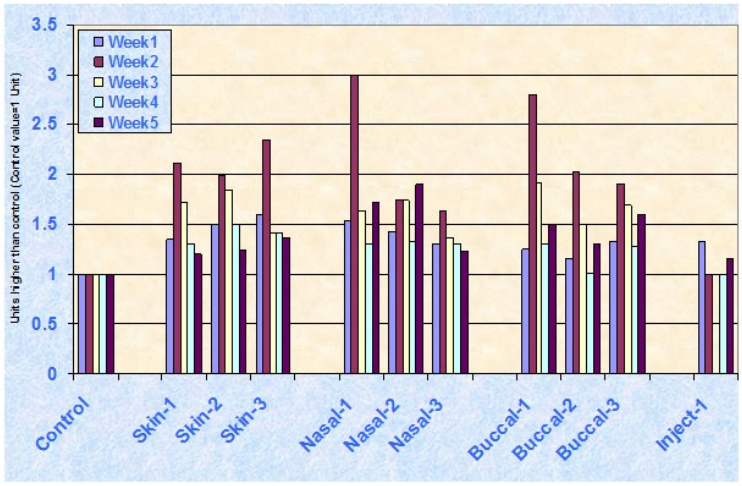

Fig 7: IL-4 response in sensitized animals

A positive response was clearly visible following sensitization through skin, nasal and buccal routes. The response was peaked in Wk-2 onwards, 2 to 3x higher than controls. No marked differences were visible with the different doses of antigen when sensitized through skin, however, nasal and buccal routes showed significantly higher response with 20 ug per dose sensitization. There was slight response in the SC-Inj route.

IFN-γ response

Fig 8 shows the IFN-y responses in all routes of sensitization. Nasal and buccal routes showed 1.5x to 2x higher than controls.

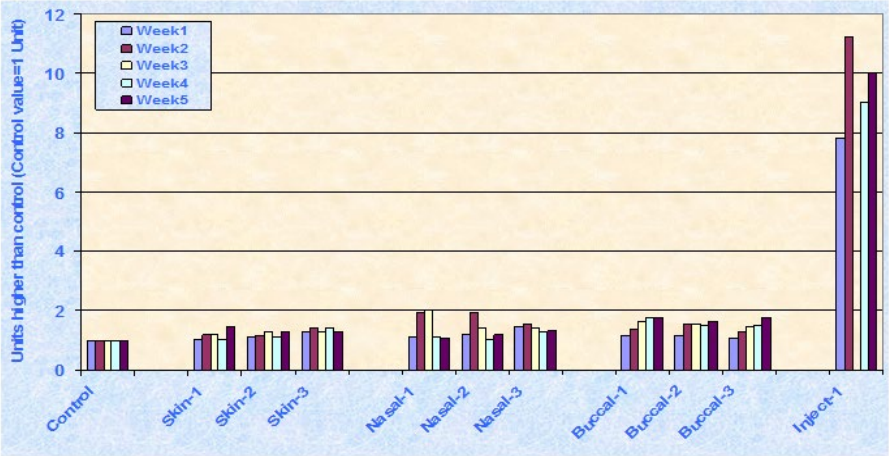

Fig 8: IFN-gamma in sensitized animals

A sustained response, higher than the control was seen in nasal group. The response was very marked and consistently higher as time lapsed in the buccal route. The SC route showed a very acute response ie, 8 to 10x higher than controls.

Route	Skin	Nasal	Buccal	S/C inj
IgM	Wk-3 onwards 1.5x higher	Not appreciable	Wk-3 onwards 1.5x higher	Appreciable 1.5-2.0 higher
IgG	Wk-2 onwards 2-2.5x higher	Low	Wk-2 onwards 2.5x higher	Wk-2 onwards 2-2.5x higher
IgG_1	Wk-4 onwards Slight	Slight	Wk-4 onwards 2-4x higher	Wk-4 onwards
IgG_{2a}	Wk-4 onwards 2x higher	Low	Wk-4 onwards 2-3x higher	Wk-2 onwards 2x higher
IgA	Not appreciable	Appreciable 1.5x higher	Slight	Wk-2 onwards 2x higher
Dose relation	No	Lower dose, better response	Dose related	Dose related

Table 1: Summary of antibody response in mice sensitized with leishmanial exo-antigens through different routes

Discussion

Three groups of nine female BALB/c mice were exposed to antigen. In the first group antigen was applied to the dorsal surface of the pinna. The second and third groups had the antigen delivered into their buccal or nasal cavity, respectively. Three concentrations of antigen were utilized for the experiment and three mice in each group were individually exposed to each antigen concentration. All antigen applications were made without the use of adjuvant. Saliva and plasma samples were obtained each week for six weeks and antibody levels (IgM, total IgG, IgG1, IgG2a and salivary IgA) measured with an ELISA utilizing the same batch of antigen as that to which the mice had been exposed. Our results show that application of microgram quantities of *L. donovani* -Exo- antigen to intact mucous membrane or skin of mice, without the use of adjuvant, can lead to specific antibody production and cytokine response. The buccal mucosa was the most efficient route for sensitization. This finding that antigen delivery into the buccal cavity generated the highest antibody levels holds particular relevance in animals where grooming can lead to increased buccal exposure. Consequently, antibody prevalence rates may not always reflect the true infection rates particularly in canine leishmaniasis.

These data highlight the need for reliable antigen-detection systems.

An immunogen, when administered in absence of accompanying adjuvant or immuno-modulating vehicle, if it generates a specific and potent response which is of diverse in nature, then it shows its potency in stimulating the immune response. It also reflects on the practical utility of its application for modulating the immune response. Furthermore, generation of diverse immune response means the strategy followed in sensitization has certain advantages and may be useful for planning effective immunization. It is well established how T- and B-cells react to the presence of non-self antigens. After contact with antigen, these cells are activated and undergo differentiation and respond to various signals. The induction of an antibody response involves more than an interaction between antigen and specifically reactive cells. Ancillary cells such as dendritic cells, macrophages and granulocytes phagocytose and sequester antigen and via MHC convey to T-cells and B-cells. In addition, serum factors such as complement also aid in modifying the antigen thereby specific antibody response will be inducted. Antibody responses may be T-cell dependent or independent.

L. donovani Exo-antigen qualifies for this and therefore selected for the sensitization experiment. No adjuvant or any other molecule which would stimulates the immune system was used. The diluent used was the blank antigen medium. Only antigen alone was used. Three routes, - cutaneous, nasal, and buccal routes were chosen to deliver the antigen. The antigen doses were concentrated to obtain 20 µg, 100 µg and 500 µg in 25 µl volume. There was no correlation of the response to the antigen dose. Reasons could be that as antigen applied topically and also in situ, there is some limit for the antigen to adsorb into the system. The same degree of response was evident with 20 µg level.

Usually secondary antibody responses primarily of IgG and its subtypes are known to be T-cell dependent whereas IgM response could be dependent or independent of T-cells. The T-cell independent IgM responses are usually of low affinity and has low efficiency in antigen binding site. The circulating IgM being polyvalent, may show functional affinity or avidity. In our ELISA, it is quite possible that we have detected the circulating polyvalent IgM antibodies against *L. donovani* Exo-antigen.

There are indications that IgA may be similar to IgM in being relatively T-cell independent and of low combining site affinity whereas IgE may be similar to IgG in being data dependent and of high combining-site affinity. However, no clear data is available however on T-cell dependence or independence of circulating IgA antibody production.

A number of factors associated with *L. donovani* Exo-antigen.

antigens influence the outcome of immune response. If sensitization is done with LPS dominant antigens, then predominant IgM antibody response and down regulated DTH response is expected. These antigens do not stimulate T-cells at all. Antigens known to be T-cell dependent induce IgG response in vivo.

Based on the nature of sensitizing antigen it is expected of the induction of immune response. The *L. donovani* Exo-antigen is of polymeric type, naturally it is expected to generate increased efficiency of immune response. With the result of high affinity antibodies in plasma and cytokines due to the activated T-cells and B-cells. T-cells are primed more efficiently with small doses of antigens. With increasing the antigen dose, the mitogenic functions of ancillary cells increase which invariably boost the B-cells. Increase in antigen dose converts a "no-signal" to "on-signal" situation. (here the IgM is seen more with higher antigen groups. T-cells reduce the availability of antigen for B-cells and hyperactive T-cells would prevent the expression of antibody formation in some B-cells. This may be the scenario in SC-Inj group where excess IFN-y produced with no obvious IgG2a.

No adjuvant or any other immuno-stimulatory material was used. Amongst the routes used, the buccal route was found to be most sensitive, then nasal and cutaneous route. Sensitization with as low as 20 µg dose of promastigote antigen induced appreciable level of total IgG, IgG1 and IgG2a compared to control animals. This shows that antigen sensitization is very effective through the contact with mucous membrane. The cytokines IL-4 and IFN-y were also generated following sensitization indicating that promastigote antigen can elicit both Th-1 and Th-2 responses. As IFN-y level was significant, then it was suggestive that promastigote elicit a protective Th-1 response readily without any use of adjuvant. Contact with a micro-amount of antigen was enough to trigger immunological responses for seroconversion and mucous membrane contact seems to be critical. On the whole, the promastigote antigen has elicited a potent anti- *Leishmania* response. High leishmania antibody levels are sometimes found in healthy residents of leishmania-endemic regions. The basis for their seropositivity is unknown but is frequently attributed to previous infections, a sub-clinical infection, or cross-reactivity with antibodies to other co-endemic diseases. However, this finding has practical implications because antibody-based assays are traditionally used to estimate disease prevalence and assess risk in human and canine populations. Moreover, if antibody levels are unlinked to infection and can occur with inadvertent exposure to parasite antigen, then the reliability of antibody-based tests for diagnosis and screening could be compromised in specific instances. A study was, therefore, undertaken to determine whether exposure to promastigote soluble exo-antigen alone could lead to antibody production in the absence of infective parasites.

In BALB/c mice, macrophages lack expression of MHC associated recognition molecules (Handman et al.,1979). Production of high affinity antibodies (in terms of high titre) is dependent upon the presence of activated T-cells by the sensitized antigen. Mucosal immunization has considerable amount of implications. The biomass of mucous space in the host is multitude larger than any finite area such as skin or any parenteral site. As our work showed a strong immune response across the mucous route, there will be an excellent scope for planning protective immunization through this route. The mucous membrane route is found to be sensitive and generates highest response with low antigen dose. A cost-effective delivery can be anticipated through this route. In addition, unlike the traditional route, the mucous membrane route may be an effective way stimulating IgA antibody response more effective than other routes such as skin. The log response to the unit of surface area works efficiently through the mucous membrane (buccal, nasal, SC-Inj routes). Our data show effectiveness of mucous membrane sensitization in generating both humoral and cytokine response. This is what happened with *Leishmania* soluble antigen. Normally several adjuvants such as CFA, glucan, BCG, or liposomes are usually used to enhance the immune response. A diverse immune response was stimulated following sensitization with promastigote antigens. In addition, animals were also exposed to different doses of in vitro derived antigens from promastigotes by different routes. We demonstrated a diverse antibody and cytokine response soon after sensitization. Results have shown that an *in situ* delivery of the antigens on to the mucous membrane has resulted with aggressive immune response. The response has been modulated towards Th-1 type.

The novel homologous promastigote antigens of *L. donovani* parasites free from serum and other essential proteins normally used under in vitro culture conditions were used to sensitize the BALB/c mice by

various routes. It is known that cytokine responses are upregulated with the use of adjuvants, prolonged release of antigen, migration of effector cells and consequent mitogenic effect. These factors coupled with genetic factors would generate selective cytokine response. A variety of strategy has followed to induce protective immune response, for example soluble leishmania antigen with recombinant IL-12 is known to protect BALB/c against experimental *L. major* infection. Recently Stager et al. (2000) has shown that recombinant hydrophilic acylated stage–regulated surface protein B1 antigen of *L. donovani* designated rHASB1, is known to elicit Th-1 response in absence of adjuvant and generate IL-12 in the dendritic cells, and rHASPB1-specific IFN-y producing CD8(+) T-cells.

Several types of antigens such as flagellar protein alpha- and ss-tubulin, histone H2b, ribosomal protein S4, malate dehydrogenase, elongation factor 2 were used to induce parasite-specific T-cell response (Probst et al. 2001). A striking T-cell response was evident with histone H2b antigens. Recently Kamhawi et al. (2000) have reported prior exposure of mice to the bites of *Phlebotomus papatasi* has conferred powerful protection against *L. major* infection associated with a strong delayed-type of hypersen sitivity response and with IFN-y production at the sire of parasite delivery. These results have important implications epidemiological consequence.

We have adopted novel application of antigen by different routes. Then such an antigen would have considerable promise for regulating the effective protective response. The main objective of this study is to study how a naïve individual would respond to the exposure of soluble *Leishmania* antigen. This work has showed that the soluble *Leishmania* immunogen possesses the ability to stimulate immunoglobulin synthesis within the body and also IgA synthesis in mucosal system. This would be useful in monitoring immune response when soldiers are exposed to the vector-bites during their combat and field operations. If there is an exposure, either in the form of vector bites, or by occupational means, it is interesting to know how one would react to the antigen? Would it generate any allergic or hypersensitivity reactions? Which type of antibodies are normally expected following sensitization. How do they respond immunologically? As mentioned earlier, there are some unexplained ways people have responded to the leishmanial antigens; some have not even visited the endemic areas and absolutely no evidence of vector bites.

The BALB/c mice being a non-healer type seem to possess a recessive Th-1 response and a dominant Th-2 response. These responses observed under experimental conditions may be useful in understanding the immune response in humans under endemic situations. After antigen sensitization, dendritic cells are stimulated and releasing IL-12 cytokine and initiate Th-1 response and also stimulate the Langerhans cells which upregulate MHC class 1 and class II antigens with the predominant response of IFN-y (von Stebut et al., 2000).

There have been several studies conducted on the presentation of antigen through trans-cutaneous (TC) route (Glenn et al 1998b). All these studies have clearly established that TC route [Scharton-Kerston et al (1999)] is a very effective for vaccination thereby the immune system can be stimulated. Major component of skin, the epidermal Langerhans cells migrate into the skin from bone marrow, phagocytose antigen and then migrate into the draining lymph node. The predominant antigen presenting cell, the epidermal Langerhans cell, has shown to possess a constant level of to the draining lymph node. Two locally produced cytokines TNF-α and IL-β appear to play dominant roles in activation and subsequent migration of LC out of the skin (Stager et al., 2000). Recently Giraud et al. (2018) showed the effect of dermal route and investigated mouse ear-skin response to promastigote secretory gel and find that a significant proportion of up-regulated transcripts are involved in inflammation and cell recruitment.

In an endemic area, there are more people sero-converted than the people showing clinical form of disease (Badaro et al. 1986). Based on the epidemiological tool, there may be lot more people sensitized than the sero-converted. Some general observations were made with the laboratory workers where they became sero-converters having worked more closely with the parasite culture and by handling the antigens. And also other people involved in the field work or otherwise come in contact with the leishmanial antigen.

An experimental approach would provide some explanations for the above-mentioned situations. It is hoped that some accurate information on the nature of response would emerge from the controlled study in animals. Such information would be useful to explain the situation in humans. Essential requisite for such studies is the availability of parasite specific and immunologically active, critical antigens free from any other proteins. A novel method of harvesting such critical antigens designated "homologous promastigote antigens" has been developed at the Walter Reed Army Medical Centre. The experiment is designed to expose the susceptible group BALB/c mice to leishmanial antigen at three levels by different routes. It was intended to use a leishmanial antigen to study the systemic response.

With this scenario, five categories of people are expected:

(a) exposed but not infected (resistant individual, in other words, endemic normal),

(b) exposed, with sub-clinical infection (susceptible individual before patency or preclinical condition),

(c) exposed and showing clinical infection (susceptible individual showing patently active infection).

(d) Under field conditions, the group (c) individuals may receive chemotherapeutic treatment and form an additional group, infected and treated group (Group D).

(e) Normal individuals in non-endemic area serve as unexposed or naïve controls (Group E).

How could one differentiate between the first three categories of people under field conditions? The immunological response elicited after infection may provide an insight into the differentiation of these four groups. Hopefully differentiation can be done based on the type of specific antibody response and with the parasite specific T-cell responses. This would certainly benefit the disease monitoring under field conditions. It is logical to see the endemic residents either susceptible to or resistant to infection. In a given endemic scenario, usually a large number of residents remain free from clinical conditions. There may be some degree of natural resistance operating in them (Group a and b). However, only a small percentage of individuals may show some degree of clinical conditions (Group c). There could be acute and chronic situations. In the proposed experimental work, we hope to understand some possible indicators for differentiating these groups. The information gathered would provide some key indicators for differentiating the soldiers and military personnel participating in the combat operations in the endemic areas.

As early as 1974, Radwanski et al. have demonstrated presence of IgG2 in guinea pig model by IFA technique. It is known that antibody acts as a potentiator for macrophage activation. Later on, anti-*leishmania* antibody and leishmanial antigen-antibody complexes are shown in cytotoxicity studies (Stager et al., 2000). It was suggested that antibody might play a part in conjunction with CMI response in the recovery from infection. Our studies show that buccal route is very

useful in sensitizing against *leishmania* parasites. Buccal route has been used as a means of immunization and vaccine delivery in several viral and bacterial infection (Kraan et al., 2014).

The role of Th-1 or Th-2 response in determining resistance or susceptibility to infection has been well documented with experimental situation in genetically determined inbred mice. Being susceptible to *Leishmania* infection, the BALB/c mice, possesses certain ability to mount a vigorous Th-2 response associated with production of IgG1 antibody and IL-4 and IL-10 cytokines. However, it has no ability to mount a Th-1 response equivalent to that could be seen in resistant animals such as, C3H/He, characterised with no significant levels of IgG2a antibody (being the cardinal features of Th-1 cellular response) and no IFN-y and IL-12 cytokine production. IL-4 production down regulates the Th-1 type of response. In recent years, humoral response is very well recognized and has several applications. In humans, the role of cellular response of Th-1 type in the protection or resolution of infection is well established in cutaneous leishmaniasis. An impairment of IFN-y production, high IL-4 and IL-10 levels are essential features of Th-2 response. This polar response classically identified in genetically defined mice. It is known that BALB/c mice is unable to elicit Th-1 response due to its genetic background. This feature may change as per the manipulation of immune activation. In our case, buccal exposure of antigen obviously shown to elicit Th-1 response even in BALB/c mice. This could be used as a means of delivering *L. donovani* immunogen.

There could be acute and chronic situations. In the proposed experimental work, we hope to understand some possible indicators for differentiating these groups. The information gathered would provide some key indicators for differentiating the soldiers and military personnel participating in the combat operations in the endemic areas. Infection with amastigotes of an atypical strain UR6 is unable to undergo viseralization in hamster and BALB/c mice and therefore examined as a means of immunoprophylaxis without any adjuvant. Such active-prophylaxis with amastigotes has generated high level of IgG2a in BALB/c mice (Mukhopadhyay et al.1998; 2000).

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