Effect of immunization with lipid associated polysaccharide antigen and anti CD-2 antibodies on class II MHC expression and cellular immune response in BALB/C mice infected with *Leishmania donovani*

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Visceral leishmaniasis (VL) infection caused by *Leishmania donovani* is yet to find a satisfactory cure in absence of suitable chemotherapy. Clinical resistance for anti Leishmanial drugs is now frequently encountered among patients of Kala-azar cured patients provide considerable impedus for in absence of suitable chemotherapy. Clinical

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The present study describes the result on the role of lipid associated polysaccharide antigen of L.donovani for immunogenicity and in modulating the response of antigen presenting cells required for activation of cellular immune response against challenged infection in BALB/C mice. To promote the ability of these antigens in achieving immunity, mAb to CD-2 antigen was used as adjuvant and efficient carrier abilities of this formulation was evaluated and compared with other formulations (FIA, Saline).

Material and Methods

Parasite—Leishmania donovani strain DD8 (MHOM/IN/80/DD8) was propagated in medium M-199 (GIBCO, Grand Island, New York) supplemented with 20% heat inactivated fetal calf serum, 12mm HEPES, 50 U/ml each of penicillin and streptomycin, and 80 U/ml of gentamicyn. Promastigotes were regularly subcultured every 72 hrs in this medium.

Antigen preparation and immunogen—Lipid - A associated polysaccharide (LPS) was isolated as previously described7. Briefly, promastigotes harvested from Log phase culture were centrifuged (2000 rpm x 10 min.) and sedimented pellets suspended in lysis buffer [6m guanidine hydrochloride (GSH), 15m EDTA (MERCK), 1mm mercaptoethanol (BDH)] were cooled to −15°C. Detergent, Tween-80 was added to obtain good suspension of Leishmania required for effective lysis. After physical treatment, the lysed suspension was incubated at 4°C (1hr). Following centrifugation (15000g x 10 min, 4°C), the lysate was extracted with Butanol to yield LAP-LPS. After hot phenol water extraction, protein free LPS, as confirmed by SDS PAGE and protein estimation, were isolated from water phase and partially purified LAP protein from phenol phase. The antigen solution (15 μg/100μl PBS) was emulsified in equal volume of Freund's incomplete adjuvant/0.85% saline/1.500 diluted anti-CD2 mAb (Dakopatts A/S Denmark) in separate batches.

Immunization and challenge—Inbred BALB/C mice, 10 per group were immunized, sc with whole (1x107 per ml) or LPS antigen in 0.05ml of FIA/Saline/anti-CD2 in 1, 3, 5 and 7th week. Ten days later, animals were challenged with 1x107 promastigotes of WHO reference strain of L.donovani (MHOM/IN/80/DD8). The challenged mice were monitored till day 42. Protection induced was evaluated by observing the survival rates.

Specific antibody response—As an approach to evaluate the immune mechanism responsible for immunity induced in immunized mice, the kinetics of antibody response was determined on day 28 and day 40 by Direct Agglutination Test (DAT)8.

Post immunization macrophage and T-cell changes—To query the kinetics of activation of cellular factors required for protection, changes in behaviour pattern of macrophage was studied for expression of Class II MHC antigen by ABC-Immunoperoxidase staining using mouse monoclonal antibody to Class-II antigen9. The ability of macrophage in association with MHC-Class-II to support T-cell activation was also studied by setting T-cell culture with monocytes and later estimating the supernatants for the release of Interleukin-210 and MIF11. For this, spleen cells were isolated from BALB/c mice by homegenizing spleens with 0.15 M phosphate buffered saline (PBS). Cells (1-2x106/ml) were suspended in medium RPMI-1640 (GIBCO) supplemented with 10% heat inactivated fetal calf serum (FCS), 12 mm HEPES and 50 U/ml of gentamicyn. Cells were allowed to adhere to 22-mm glass coverslips at 37°C in 5% CO2 in air. Three hours after plating, coverslips were washed with RPMI-1640 medium to remove non adherent cells and were reincubated in fresh FCS-supplemented RPMI medium with mouse monoclonal antibody to MHC-DR for 30 minutes. After blocking the peroxidase of the washed cells with 0.3% H2O2, Avidin peroxidase (1:100 in TBS) was added and subsequently, cells were stained with DAB (0.5 mg/ml TBS) to observe Class-II MHC expressed on surface.

Later, the T-cells from nonadherent cells were fractionated from B-cells using nylon wool column and cultured in presence of 20 μg/ml Leishmania donovani antigen for 48 hours (37 deg. C, 5% CO2 in air). These antigen primed immune T-cells were transferred to plate for their coinubcation with macrophage for 24 hr (37°C, 5% CO2 in air). For IL-2, 75 μl of supernatant of macrophage T-cells culture was incubated into wells of micro titre plate in final volume of 0.2 ml for 20 hrs. Later, the cells were incubated in presence of 20 μl of 2.5 mg/ml MTT (37°C, 4 Hrs.). Isopropanol (100 μl) was added to dissolve blue formazon crystal and finally IL-2 release was determined in ELISA reader at a test wavelength of 570 nm.

Statistical analysis—The significance of difference in antibody levels and monocyte MHC-II expression among different formulation were evaluated by two
way analysis of variance. The significance of difference in antibodies level and MIF on day 28 and 40 was determined by paired t-test.

Results

Immuno-protection of leishmania lipid associated polysaccharide—The protection afforded in experimental formulations against challenge with L. donovani was determined in comparison to experimental formulations and control. Mice immunized with lipid associated polysaccharide of Leishmania with anti CD-2 demonstrated 95% survival which was significantly higher (P<0.01) than the control group (40%). Further detailed studies were carried out on their immuno-reactivity as described in succeeding sections.

Specific antibody response at different time points after immunization—Whole/Specific antibody response induced by LPS complexed in anti CD-2 antibody against challenge with lethal dose of L. donovani was determined in comparison to other formulations and control. The group immunized with LPS antigen linked to anti CD-2 antibody induced a higher antibody response in 100% mice (P<0.005) on day 28, compared to their counterparts immunized with FIA and 0.85% saline. On the other hand, the Whole Leishmania immunized group also demonstrated high antibody titre in combination with this putative adjuvant in 90% mice which was significantly higher (P<0.01) than the control group (60%) or when used with other putative adjuvants (FIA-70% in Saline 65%). On day 40, antibody titre declined in 100% mice immunized with LPS-anti-CD-2 which possibly indicated shifting of immunized animals from infection towards recovery (Fig. 1). The above decline in antibody titre following immunization with whole promastigote antigen was in 80% mice in anti CD-2 adjuvant and in 20% mice with FIA. No decline in titre was observed in mice following immunization with whole promastigotes and 0.85% saline.

Macrophage response to whole or LPS antigen—Response of macrophages for expression of class II MHC to whole or LPS antigen in combination with the three putative adjuvants was examined as described earlier. The macrophage function for MHC-II expression observed in group immunized with LPS antigen in combination with anti-CD-2 antibody was maximum (40-45%) on day 40 post challenge (Fig. 2). This response was significantly higher (p<0.001) than that of infected control mice (10%) and LPS-Saline (17-20%). Corresponding value for MHC-II expression in mice vaccinated with whole promastigotes linked to anti-CD-2 was 20-22% which went further low when whole antigen was linked to FIA (15-17%) or saline.

Cytokine response to whole or LPS antigen—Increased macrophage function in LPS-anti CD2 immunized hosts was later observed associated with raised T-cell function for MIF response (Fig. 3). MIF release up to 55% was observed in correspondence to 40-45% macrophage showing expression for MHC-II molecule in these animals. The above response was however not observed in other formulation where number of macrophages expressing class II MHC-molecule was low (whole antigen with anti CD-2: macrophage-MHC-II 20-22%, MIF 30%). Further effect of LPS in induction of protective immunity was observed in enhanced IL-2 release response (Fig.4) as

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Fig. 1—Decline in anti -L. donovani antibody titre, day 40 post immunization with different Ld. Antigen preparation in immunized host.

Fig. 2—Effect of lipid associated polysaccharide immunization with CD-2 on class ii MHC expression in balbc macrophage challenged with L. donovani.
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Discussion
The results reported here show that lipid associated polysaccharide (LPS) extracted from promastigotes of Leishmania donovani when used as immunogen can reduce the immuno-suppression induced by this parasite in susceptible BALB/C mice. Our results are in agreement with previous reports on immunogenic properties of this component which shows abundant presence on surface of Leishmania species. We also observed that LPS required the presence of anti-CD-2 antibody as carrier which efficiently promoted the ability of LPS in achieving immunity to Leishmanial infection.

To characterize the role of LPS and immunization mechanism which may be used to produce a protective response, we examined the expression of class II MHC in BALB/C macrophage and later examined its effect on IL-2 and MIF response of T-cells. The MHC class II expression was observed upregulated in LPS immunized mice and infected macrophages possibly presented the antigen to Th1 cells, which was obvious from increased IL-2 and MIF response. The immunization protocol presented in this study reflects that antigen presentation from activated macrophages is important after which LPS antigen may be effective in re-stimulating T-cells primed by previous immunization. We used DR antigen to study the expression of class II MHC in BALB/C macrophages because similar structural variations have previously been shown between mouse and human class II MHC molecule and reports on human DR genes to be counterpart to mouse IE genes due to analogy are also available.

The inability of most vaccine formulations to prevent the infection has been the feature observed for most experimental vaccines against Leishmaniasis so far. Hence, evidence for the role of LPS in immunity to Leishmania donovani infection shown in this study is worth pursuing.

We especially looked for a role of anti-CD-2 as new adjuvants since trials with BCG, vaccinia virus, salmonella, LACK + IL-12 and C.Parvum, formulated in different membrane constructs of Leishmania have not been observed very successful. Besides, alum was also reported inefficient while MF-59, IL-12 DNA and IL-12 DNA are still under trial with inconsistent results.

It is not surprising that anti-CD-2 proved as effective adjuvant to LPS given the fact that CD-2 on T-cells are involved in pathways to regulate protein kinase-c, that induces the expression of various activation genes of Th1 cytokines including the genes of IL-2 and IL-2 receptors. Role of CD-2 in generation of co-stimulation signals during interaction of T-cells with antigen presenting cells has been shown by T-cells in mice immunised with LPS linked to anti-CD-2 antibody, which was comparable with that of infected control. The effect of LPS on IL-2 response with other adjuvants was comparatively low with FIA (1.10-1.12) and 0.85% saline (0.98). Corresponding IL-2 value in mice immunized with whole antigen further decreased with anti-CD-2 (1.0-1.3), FIA (1.00) and saline (0.78-0.80).

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shown besides reports of its functional association with CD-44 and CD-45.39

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