SUMMARY

Visceral leishmaniasis (VL) constitutes one of the major tropical disease in India. Important contributing factors in the spread of the disease are: (i) failure to detect the parasite in the early stage of infection; (ii) poor understanding of basic mechanisms responsible for pathogenesis of the disease and; (iii) attainment of drug resistanceby the *Leishmania* parasite. *Leishmania donovani*, the causative agent of VL, resides as amastigotes within the phagolysosomes of host macrophages and exerts the clinical manifestation of the disease. Intracellular localization of the parasite further hampers the anti-leishmanial chemotherapy.

Studies on the surface molecules of *Leishmania* parasite and infected macrophages are of central importance in understanding basic mechanisms involved in pathobiology of the disease and can also aid in identifying the targets for therapeutic interventions. The leishmanial components, associated with the promastigotes, amastigotes and infected macrophages, have been mainly studied utilizing; polyclonal and monoclonal antibodies (MAbs) generated by heterologous immunizations, patient sera, excretory factors, enzyme activities and metabolic/surface radiolabeling. The antigens, so identified, are usually immunodominant and also cross-reacted with other pathogenic micro-organisms.

Utilizing homologous antiserum raised against malaria-infected cell membranes, recent finding in the lab conclusively demonstrated the efficacy of the approach in identifying infected cell surface determinants. In the present study, work was further extended to *Leishmania*-infected macrophages. The objective was to utilize such an approach to identify clinically relevant 'minor' antigens of *Leishmania* which are otherwise not detectable by conventionally raised anti-parasite antiserum. For this, polyclonal antiserum and monoclonal antibodies were generated by immunizing either *in vitro L. donovani*-infected macrophage (4 h post infection) membranes or heat-killed promastigotes. These antibodies were used to compare the antigenic profile of *Leishmania* promastigotes, 'amastigote-like' forms and infected macrophages. Antibodies were subsequently checked for their inhibition of *in vitro* growth of parasite and macrophage invasion.

Anti-infected macrophage membrane (anti-IMm) antiserum (strain RMRI68) recognized both infected cell surface and promastigotes as revealed by immunofluorescence assay (IFA), flow cytometry and immunoelectron microscopy (IEM). The antiserum generated against homologous normal macrophage membranes did not show any reactivity with infected macrophages. Also, anti-IMm antiserum did not react

with *in vitro* cultured normal macrophages. This established that the normal macrophage membrane components were neither immunogenic nor participated in the binding of the antisera. Anti-promastigote (Pr) antiserum recognized the intracellular parasites without any apparent reactivity with the infected cell surface. Anti-Pr antisera, unlike anti-IMm antiserum reacted well with LPG-KMP11 and gp63 molecules of *Leishmania*. Using live promastigotes of three strains of *L. donovani* (RMRI68, AG83, DD8) it was observed that the anti-Pr antisera cross-reacted with all the three isolates. While, anti-IMm antiserum specifically recognized the corresponding strain used for macrophage infection.

In immunoblotting, anti-IMm antiserum recognized 160, 140, 120, 72, 64, 58 and 43 kDa antigens in infected membrane and 90, 84, 82, 74, 46, 42, 38 and 20 kDa antigens of promastigotes. On the other hand, anti-Pr antiserum reacted with 62 and 44 kDa antigens of infected macrophage membrane and 90, 42, 38 and 20 kDa antigens of the promastigotes. Immunoprecipitation with live [^{35}S]methionine-labeled promastigotes revealed the recognition of 34, 32 kDa and two protein of mol. wts. < 14 kDa by anti-IMm antiserum.

Fusion of PAI-O myeloma cells with spleen cells from mice immunized with homologous infected macrophage membranes or heat killed promastigotes resulted in twelve clones of hybridomas producing antibodies reactive with promastigotes, 'amastigote-like' forms and infected macrophage membrane antigens. All these antibodies were directed to different epitopes as revealed in multiple tests including ELISA, IFA, flow cytometry, IEM and immunoblotting.

Important observations based upon their reactivity patterns are:

(i) all MAbs, except MAb RmE9D7, were positive with glutaraldehyde-fixed promastigotes in ELISA. RmB2F8 reacted with the promastigotes only in ELISA and not in other tests.

(ii) five MAbs; RpE4C10, RpE2E7, RmE3D6, RmE9D7 and RmF5G3 revealed strong fluorescence with air dried/acetone-fixed promastigotes, indicating a cytoplasmic localization of these antigens.

(iii) epitopes recognized by MAbs RmB4G2, RmB2F8, RpB2F10, AmE6E8 and AmF5F8 were sensitive to metaperiodate treatment, indicating the involvement of carbohydrate

moieties. This was further confirmed by their positive reactivity with LPG-KMP11 complex. Two MAbs RmC8D6 and RpE2E7 revealed positivity with gp63 molecule.

(iv) seven MAbs; RmB4G2, RmC8D6, RpE4C10, RpB2F10, RmF5G3, AmE6E8 and AmF5F8 agglutinated the promastigotes. MAb RmE3D6 reacted with live promastigotes but without any apparent agglutination while, RmF5G3 recognized the promastigotes in all assays.

(v) promastigote antigens identified by MAbs RmC8D6, RmF5G3, RmE3D6 and RmE9D7 were < 29; < 29; 44 and 49 kDa respectively. While, MAbs RpE4C10, RpB2F10 and RmD9B8 exhibited the recognition of multiple polypeptide bands of promastigotes.

vi) MAb RmC8D6 was found to be promastigote specific and did not react with infected macrophage as revealed by different tests.

(vii) in IFA MAbs RpE4C10, RpE2E7, RmE3D6, RmF5G3 and RmE9D7, which were reactive with promastigotes and intracellular parasites, also recognized the 'amastigote-like' forms. MAb RpE4C10, RpE2E7 and RmE9D7 did not recognize any antigen of 'amastigote-like' form in immunoblotting while MAbs RmE3D6 and RmF5G3 reacted with polypeptide bands of mol. wts. 51 and < 29 kDa respectively. Another MAb RmB2F8, which was negative in IFA with 'amastigote-like' form, recognized a 50 kDa antigen in immunoblotting.

(viii) MAbs RpB2F10, RmE3D6 and RmF5G3 reacted with paraformaldehyde-fixed macrophages (in IFA, flow cytometry and IEM) and the antigens recognized in crude membranes of infected macrophages were 84; 84 and 72 and; < 29 kDa respectively. It is important to note that these MAbs were also reactive with the promastigote surface.

(ix) only one MAb RmE3D6 reacted with the paraformaldehyde-fixed normal macrophages in flow cytometry and recognized 56 and 100 kDa antigens of the normal macrophage membrane.

(x) using macrophage membrane preparation from strain RMRI68 infected macrophages or strain AG83-infected macrophages, it was revealed that six MAbs; RmB4G2, RpB2F10, RmE3D6, RmD9B8, RmF5G3 and AmE6E8 identified polypeptide bands in

immunoblotting. Antigens recognized by RpB2F10, RmD9B8 and RmF5G3 were specific to strain RMRI68 infected macrophage membranes. Two MAbs RmB4G2 recognized common antigen of <29 kDa. While, MAb AmE6E8, raised against *L. donovani* (strain AG83)-infected macrophage membranes, identified strain-specific antigen of mol. wt. < 29 kDa in corresponding infected macrophage membrane.

(xi) four MAbs; RmB4G2, RmF5G3 and AmE6E8 inhibited *in vitro* the promastigote growth as well as *in vitro* invasion of macrophages. The MAb RpB2F10 inhibited parasite growth *in vitro* without any inhibition of invasion while RmB2F10 and RmE3D6 inhibited the macrophage invasion only.

The potentially important findings of this investigation are: (a) antiserum generated by homologous immunizations with *in vitro L. donovani*-infected macrophages behaved differently towards the infected cell membrane and promastigotes; (b) unlike anti-Pr antiserum, anti-IMm antiserum exhibited the strain-specific recognition of the live promastigotes; (c) MAbs generated utilizing the above immunization strategy identified common as well as specific antigens of the parasite. Besides, the study described may form foundation for: (i) dissecting antigenic diversity among 'neo-antigenic' determinants expressed on the surface of *Leishmania*-infected macrophages and; (ii) identifying novel parasitic antigens, which are otherwise not detectable by conventional biochemical and immunological methods, but are relevant for clinical and epidemiological control of leishmaniasis and other intracellular infections.