Chapter 5
Discussion
Leishmaniasis afflicts the mankind globally with a high incidence in the tropical countries. The causative organism for the disease is a kinetoplastid, protozoan parasite belonging to the genus *Leishmania*. The parasite has a digenetic life cycle involving a free living promastigote in the sandfly midgut and an intracellular amastigote in the vertebrate host. Based on the clinical manifestations of the disease, which vary depending upon the species involved and the immune status of the host, the disease has been broadly classified into cutaneous, mucocutaneous and visceral leishmaniasis. Of these visceral leishmaniasis, caused by *L. donovani* and *L. chagasi*, is the most deadly form of the disease and is endemic in Indian subcontinent and Sudan.

Since the past two decades efforts have been focused on the parasite molecules, both shared by, and specific to, the two parasite stages. With the help of patient sera and antibodies/antisera generated by heterologous immunization of whole parasites or crude extracts, many antigenic determinants have been identified. Several of these are the immunodominant surface molecules and have been projected as candidates for taxonomic classification of the parasite and vaccine development (Dwyer, 1981; Chance, 1985; Jaffe and Zalis, 1987; Alexander and Russel, 1992; Moddaber, 1995). Additionally, identification of infection-specific antigenic determinants, displayed on the infected cell surface, is important for the better understanding of the mechanisms underlying the pathological features and clinical manifestations and may help in the development of alternative therapeutic and vaccination strategies (Singh et al., 1993; Owais et al., 1995; Miller et al., 1986; Howard and Pasloske, 1993). Recent studies in the lab have conclusively established the appearance of neo-antigenic determinants on the surface infected cells in several intracellular infections using homologous polyclonal and antisera and monoclonal antibodies generated against infected cell membrane (Choudhury et al., 1997; Majumdar et al., 2000). Also the utility of such antibodies in drug targeting (Singh et al., 1993; Owais et al., 1995) and identification of strain-specific determinants in the parasites was demonstrated (Goel et al., 1998).

In previous study in the lab (Goel, 1997) polyclonal antisera and monoclonal antibodies were generated using *L. donovani* promastigotes and by homologous immunization with *Leishmania* infected macrophage membrane. These reagents were
primarily used for the antigen characterization on the promastigote stage and infected macrophages at 4 hr post infection and several antigens common to both promastigote and infected cells were identified. It was pertinent to study the status of such antigens at later post infection time since this will yield information about the amastigote derived antigens and also help in elucidating the fate of promastigote derived antigens in the infected cells with the progression of infection. The major objective of the current study was to study the antigenic determinants in amastigotes and also present in the infected macrophages during the transformation of promastigotes to amastigotes (i.e. at the later post infection time), in order to identify antigen(s) shared by the two stages of the parasite and infected cells.

*In vitro* infection models involving mouse peritoneal macrophages, human peripheral blood derived monocytes and macrophage-like cell lines have been widely used to study the progression of infection, macrophage leishmanicidal mechanisms, elucidation of drug action, parasitophorous vacuole biogenesis, and infection derived cell surface alterations (Berman and Dwyer, 1981; Chang *et al.*, 1985; Williams *et al.*, 1986; Bogdan *et al.*, 1990). The percentage of macrophages infected remained unchanged till 48 hr post infection, suggesting that during this time the fresh cycles of infection were not initiated (*Table 1*). There was some decrease in the number of intracellular parasites after 24 hr post infection (*Fig. 2b*), probably due to the destruction of the dead parasites which might have been phagocytosed by the macrophages during infection. However, the parasites started replicating 48 hr post infection resulting in the increase in their number in the infected cells. Similar infection profile was observed by previous workers with *L. donovani*-infected human blood derived monocyte system (Pearson *et al.*, 1982; McNeely and Turco, 1990). In several studies, LPG mutants have been demonstrated to be compromised in intracellular survival within macrophages. R2D2 was found to be more efficient in infecting the human monocytes and was demonstrated to survive and multiply up to 18 hr (McNeely and Turco, 1990). The initial high rate of infection observed in the present study was in accordance with this report. However, the discrepancy observed with respect to the intracellular survival might be due to the differences in the host cells used. Additionally, unlike previous report, in this study thioglycollate-elicited mouse peritoneal macrophages were used. The peripheral blood derived monocytes are usually cultured for several days prior to infection and may thus might be considered as resting cells. However, thioglycollate-elicited peritoneal macrophages are considered to be somewhat
activated (Ogmundsdottier and Wiew, 1980), which might be the reason for the destruction of R2D2 parasites, which already lack an important functional molecule.

The flow cytometry experiments with the live infected cells indicated the presence of the antigens on the surface of infected cell (Fig. 4a). The surface localization of 44G3 and 33E8 reactive antigens was further substantiated by their presence in the crude membrane fraction of infected macrophages (Fig. 7). However, despite its strong reactivity with the infected macrophage surface, MAb 31D6 did not show any recognition of its respective 44 kDa antigen in the infected macrophage membrane when analyzed by immunoblotting. It is possibly due to the loose association of the 31D6 reactive antigen with the host cell membrane and subsequent loss during membrane preparation. The negative reactivity of antiserum raised against gp63, which is an abundant *Leishmania* surface molecule, and MAb 31D6 with the infected cell membrane rule out any possibility of contaminating molecules arising from the intracellular parasites during membrane preparation and accounting for 44G3 and 33E8 reactive epitopes in the infected cells. With the progression of infection, the level of antigens recognized by MAbs 33E8, 44G3 and 38C10 remained unchanged. Previous reports have conclusively demonstrated the presence of LPG epitopes on the infected cell surface (Handman and Goding, 1985) as early as 5-10 min of infection. The 22 kDa antigen recognized by MAb 44G3, and some polydisperse bands recognized by 33E8 were present at identical molecular weight positions in promastigote lysate immunoblots, thus confirming their parasitic origin and presence in infected macrophage membrane (Figs. 7 & 17). The binding of MAb CA7AE, which recognizes the repeating disaccharide units of LPG, was in accordance with the precious reports and decreases with the progression of infection (Figs. 4a & 5b). Williams *et al.* (1985) demonstrated an infected macrophage surface antigen, which appears 6-12 hr post infection and increases with the increase in the post infection time. Likewise, a 51 kDa antigen was reported in *L. donovani* infected macrophages which appeared 12 hr post infection and reached a steady state by 24 hr (Basu *et al.*, 1994). In the present study, the epitope recognized by MAb 8G2 was present on the infected macrophage surface at low levels at early stages of infection and its expression increased in a time dependent manner (Figs. 4a, 5a & 6a). This points towards the intracellular origin of the antigen. The appearance of the 15 kDa glycoconjugate (Williams *et al.*, 1985) and 51 kDa antigen (Basu *et al.*, 1994) at later post infection times was explained similarly. In contrast, the surface activity of 31D6, which
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was initially very strong (Figs. 4 & 5), was completely lost by 24 hr of post infection. However, the MAb continued to recognize the intracellular parasites even up to 48 hr post infection (Fig. 6). This suggests that the antigen recognized by this MAb is probably associated loosely to the macrophages during the entry of the parasites. One of the mechanisms suggested for the incorporation of parasite antigens into the host cells involves the sloughing-off of the parasite components during invasion (Kaye, 1987). Some of the LPG epitopes were suggested to appear on the infected cells via this pathway (Tolson et al., 1990). The antigen is subsequently lost with the progression of infection as result of either shedding or degradation mediated by macrophage enzymes. Another mechanism suggested to account for the presence of parasite antigens on the host cell surface is through the lysosomal degradation of parasite components and their subsequent appearance on the cell surface (Kaye, 1987). Coupled with this is the status of macrophages as professional antigen presenting cells. However, processed antigens are presented at the cells surface in association of MHC class I and II molecules. The possibility of the antigens reported in the present study following this pathway is feeble in light of the fact that *Leishmania* infection results in the down regulation and degradation of MHC molecules (Antoine et al., 1998). Moreover, the antigens recognized in the infected cell membrane by MAbs 44G3 and 33E8 range from 22-48 kDa (Fig. 7) as opposed to the low molecular weight MHC-associated peptides of the presented antigens. Furthermore, the molecular weights of these components in infected cell membrane and parasites is identical, thus ruling out any possibility of macrophage mediated degradation and presentation of these antigens.

*Leishmania* has been reported to secrete many immunodominant antigens including LPG, acid phosphatases and PPGs (Turco and Descautex, 1992; Ilg et al., 1999). In one of the studies it was demonstrated that purified LPG is capable of binding to the macrophage surface (Handman and Goding, 1985). There is a possibility that some of the antigens are secreted by the unphagocytosed promastigotes and then get associated with the plasma membrane. Immunoblot analysis of the spent culture supernatant indicated the presence of 44G3 reactive 22 kDa antigen (Fig. 9). However, the possibility of this antigen associating itself passively with the macrophage surface can be discounted based on the following observations: (i) negative reactivity of MAb 44G3 towards the crude membrane of uninfected macrophages cultured for 6 hr with the spent medium of the parasite (Fig. 10); (ii) absence of 22 kDa component in the membranes of cells incubated
with dead parasite (Fig. 11). This dependence of this antigen expression on parasite viability is unlike that observed for LPG when the formalin fixed *L. donovani* promastigotes were able to transfer LPG epitopes to the macrophage surface (Tolson *et al.*, 1990). Also, it indicates that the 22 kDa antigen is different from LPG. The antigenic bands recognized by MAb 33E8 in the infected cell membrane, on the other hand were detected in the macrophages incubated with the heat killed parasites, indicating that the appearance of these antigens, does not depend upon the viability of the parasite, as is observed for LPG (Tolson *et al.*, 1990). Despite this similarity, the 33E8 reactive components are not LPG related, since these could also be detected in the LPG deficient mutant R2D2 and its corresponding infected macrophage membrane (Figs. 13 & 14).

Some of the immunodominant glycoconjugates of *Leishmania* have been demonstrated to be present on the infected macrophages (Handman and Goding, 1985; Tolson, 1990). There is likelihood that some of the antibodies used in the present study were directed against such components. The epitopes responsible for the binding of MAbs 44G3, 33E8 and 31D6 do not involve carbohydrate moieties as suggested by the treatment of blots with sodium-meta-periodate (Fig. 12), a process that reduces the vicinal hydroxyl groups of carbohydrates (Woodward *et al.*, 1985). Although, by this process the periodate sensitivity of the epitopes recognized by other MAbs could not be determined due to the inability of the MAbs to react in immunoblotting. However, in the previous study, following this treatment to the promastigotes coated on solid support and subsequent analysis by ELISA indicated some decrease in the binding of MAb 8G2 thereby, suggesting the epitope to involve carbohydrate (Goel, 1997). The increase in the expression of 8G2 epitope on the infected macrophage surface with the increase in the post infection time was similar to the glycoconjugate antigen reported by Williams *et al.*, (1989). However, the antigen recognized by 8G2 could not be characterized due to its non-reactivity in immunoblotting.

The LPG deficient mutant R2D2 has been characterized in detail and has been demonstrated to be defective in the galactofuranose addition, which leads to the failure of the assembly of the glycan core of LPG (Huang and Turco, 1993). The MAbs which were reactive with parasite lysate in immunoblotting, (44G3, 33E8 and 31D6) exhibited enhanced reactivity with the lysates of promastigotes (Fig. 13). LPG has previously been demonstrated to mask the recognition of promastigote antigens (Karp *et al.*, 1991). In that study, the kala azar patient sera exhibited enhanced recognition of LPG deficient
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R2D2 mutant in comparison to the wild type promastigotes. This enhancement in the recognition of parasite components, observed in this study is in accordance with the observations made by Karp et al. (1991) and indicate that epitopes recognized by the MAbs do not involve LPG. Further, the membrane of macrophages infected with R2D2 also showed the presence of 44G3 and 33E8 reactive epitopes (Fig. 14), thus suggesting that neither the epitopes recognized in the infected cell membrane are LPG related/derived nor do they depend upon LPG for their presence on infected cell surface.

Recently, a 22 kDa promastigote specific protein LdARL-3A has been demonstrated in L. donovani (Cuvillier et al., 2000). This ADP-ribosylation factor-like protein was implicated in maintaining the integrity of the flagellum and localized in the plasma membrane and flagellar pocket of the promastigotes. Although, no attempts were made in that study to check secretory nature of LdARL-3A, the 44G3 reactive 22 kDa was found to be secretory in nature. Secondly, 22 kDa protein reported in this study appear to be different from that reported by Cuvillier et al. (2000) since MAb 44G3 also recognized a faint band at 70 kDa position in the amastigotes (Fig. 17), while the antibodies against the C-terminus of LdARL-3A failed to detect anything in the amastigotes (Cuvillier et al., 2000).

In vitro system does not mimic the natural infection wherein many other cellular and biochemical factors are present in the milieu of infected macrophages. These factors might force the parasite to develop some newer molecules and also to lose some previously present. Not much has been done on the animal model of PKDL. Moreover, the mechanisms underlying this dermal tropism of visceralizing strains are poorly understood. In this study, hamster infected with viscerotropic L. donovani (RMRI-68 and AG83 and DD8) were used. In this infection model, parasites could be detected in the touch smears of spleen of hamsters infected for 3 months (Fig. 3). Some of the animals infected with RMRI-68 strain developed skin lesions on the footpad and other body parts like nostrils and rump, an indication of PKDL (Fig. 3). However, significant parasite load was observed in the spleens of animals exhibiting PKDL symptoms (Fig. 3). When cultured in vitro, these parasites transformed into motile, flagellated promastigotes. The rate of transformation was faster in lesion-derived amastigotes (2-3 days) than the amastigotes derived from spleen (5-7 days). The immunofluorescence assay with the semi-thin sections of lesions and infected spleen indicated identical distribution pattern of the antigenic determinants in both the infected tissues (Fig. 15). The only exception was
the 44G3 reactive epitope, which was detected specifically in the infected lesions and not in infected spleen. Dermal tropism of the \textit{L. donovani} has been observed following the cure of infection and as a dermonodular hypopigmented nodules/lesions might appear after 2-5 years of infection (Napier and Das Gupta, 1930; Rees and Karger, 1987; Bryceson, 1996). However, the biochemical mechanisms and molecular determinants underlying this process are poorly understood. Using a similar approach Ilg \textit{et al.} (1995) demonstrated the presence of aPPG on the intracellular amastigotes present in the lesions and its secretion in the parasitophorous vacuoles. However, with the semi-thin sections used in the present study, the precise localization of the antigen within the infected cells and intracellular parasite was not possible due to the smaller size and lesser number of parasites per infected cell. Unlike \textit{L. amazonensis} amastigotes, which are larger in size and reside in communal vacuoles, the \textit{L. donovani} amastigotes are smaller and the parasitophorous vacuoles induced by this species harbors one to two parasites (Antoine, \textit{et al.}, 1998). The MAbs 33E8 and 8G2 reactive antigens could still be found associated with the infected cells while those of MAbs 38C10, 31D6, 38E7 appear to be present in the intracellular parasites.

There is a possibility that the 22 kDa antigen recognized by 44G3 is absent in both lesion and splenic amastigotes, and the observed reactivity of 44G3 in lesion sections is due to some other cross-reactive dermal antigen or some other host derived factor present on the amastigote. The presence of host derived immunoglobulins and complement factors has been demonstrated in lesion derived \textit{L. major} amastigotes (Peters \textit{et al.}, 1997). However, results obtained with the amastigotes isolated from both the sources support the fact that the 44G3 reactivity with the lesion amastigotes was indeed due to the 22 kDa antigen which is also present in the promastigotes (Figs. 16 & 17). Thus it appears that with respect to the 22 kDa antigen, the lesion amastigotes are more closer to the promastigotes. In the spleen amastigotes, MAb 44G3 exhibited faint reactivity with a 70 kDa component (Fig. 17). This antigen can again be either: (i) a cross-reactive amastigote antigen; (ii) a cross reactive antigen present in the lesion (iii) a modified form of 22 kDa antigen. In \textit{Leishmania} several stage specific components have been reported (Handman \textit{et al.}, 1981; Jaffe and McMahon-Pratt, 1983; Chang and Fong, 1982). Also some of the antigenic components undergo stage specific modifications (Sacks \textit{et al.}, 1990; McConville \textit{et al.}, 1992). The 70 kDa protein recognized by MAb 44G3 in amastigotes, disappeared with the transformation of the amastigotes to promastigotes,
while the 22 kDa antigen started appearing as the transformation progressed (Fig. 18). Also, MAb 31D6 recognized a 44 kDa antigen in the promastigotes and 55 kDa component in the spleen amastigotes (Fig. 17). These may either be two different stage specific components or may be modified forms of the same components. Despite the differences observed in the amastigotes isolated from lesions and spleen with respect to 22 kDa antigen, the promastigotes derived from both parasite populations showed no apparent differences in their reactivity towards the various MAbs (Figs. 16 & 17). Different Leishmania species exhibit differential tissue tropism. While some are harbored in the skin macrophages, others are present in the bone marrow and splenic macrophages. However, the factors governing the specificities of tissue tropism are poorly understood. To date there is only one recent report where A2/A2 rel gene product has been implicated in the visceralization of L. donovani (Zhang and Matlashewski, 2001). It remains to be seen whether the presence of the 22 kDa antigen in the lesion amastigotes is a cause or effect of the parasite tropism from spleen to skin. Keeping in view the reactivity pattern of various MAbs, it can be stated that the lesion amastigotes are closer to the promastigotes than their splenic counterparts. However, the negative reactivity of both lesion and spleen amastigotes with anti-LPG antibody CA7AE is in line with other reports which point towards the down regulation to complete absence of LPG in L. donovani amastigotes (Sarr et al., 1998)

Most of the studies on amastigotes have been hampered due to difficulties in obtaining large amounts of parasites from the infected tissues and the inability to culture this form in large amounts (Bates et al., 1992). Continuous axenic cultures have been developed for cultivation of amastigotes. However, due to the poor understanding of the mechanisms and components involved in promastigote to amastigote transformation and thus the inability to mimic the true conditions of transformation, the status of the amastigotes obtained by these methods have been subjected to a debate. Recently, Saar et al. (1998) developed a method for axenic cultivation of L. donovani amastigotes and, using several biochemical and immunochemical parameters, established the amastigotes so generated were more identical to the true amastigotes. Also, several workers have used cell-lines to cultivate the amastigotes (Bates et al. 1992). In this study, it was observed that with respect to the antigenic determinants recognized by various MAbs, both axenic and cell-derived amastigotes appeared to be different from those isolated from infected animal tissues (Figs. 20 & 21). Both types of amastigotes expressed antigens which
recognized by MAbs 44G3, 33E8 and 31D6 and produced a reactivity profile similar to that obtained with promastigotes (Fig. 21). Even in ELISA, the reactivity of both the axenic and the cell-derived forms was intermediate to that observed for respective amastigotes and promastigotes (Fig. 20). Neither cell derived, nor axenic amastigotes exhibited 70 kDa or 55 kDa components which were recognized by MAbs 44G3 and 31D6, respectively in the spleen isolated amastigotes. Thus, in terms of the antigenic profile, the axenic and cell derived amastigotes are more promastigote like. It has been earlier reported that despite the similarity in the expression of several heat shock proteins, there is some difference in the protein profile of axenic heat induced amastigotes and tissue isolated amastigotes (Alcina and Fresno, 1988).

In *Leishmania*, it is well established that promastigotes and amastigotes employ different invasion and survival strategies in the mammalian hosts and ultimately it is the intracellular amastigote stage that is responsible for the propagation of the parasite in the host. But, infection in the natural course is initiated invariably by the promastigotes. It is possible that the 22 kDa antigen is important in the establishment of infection by the promastigotes. Using a similar approach, pretreatment of the promastigotes with anti-gp63 antibodies was demonstrated to affect the intracellular survival (Kweider *et al.*, 1987). Also, several antibodies directed against the abundant surface glycoconjugate LPG were demonstrated to inhibit the invasion of the macrophages *in vitro* by the promastigotes (Handman and Hocking, 1982). That the epitopes recognized by the MAbs are involved in the attachment of the promastigotes to the macrophages is supported by the invasion inhibition assays in presence of MAbs (Goel, 1997) or using MAb pretreated promastigotes (Fig 23). This inhibition was maximum with MAb 33E8. However, the pretreatment of the parasites with MAb 44G3 resulted in maximum impairment of the growth of the intracellular parasites (Fig. 22). Thus, it appears that the 22 kDa antigen is important for the intracellular survival of the parasites. However, its absence in the amastigote stages can be argued against this.

Many species- and strain-specific antigens have been described in *Leishmania* (Jaffe *et al.*, 1984; McConville *et al.*, 1990a; Sacks, 1992). However, most of the antigens of *Leishmania* are cross-reactive and shared among the species (Gardiner *et al.*, 1984; Lemerse *et al.*, 1985). While the strain- and species-specific antigens are important for studying differential tissue tropism, diagnosis, epidemiology and taxonomic classification of the parasite, the cross reactive antigens are likely to be good candidates.
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for vaccination and therapeutic strategies (Jaffe et al., 1984; Handman 2000). In this study, cross-reactivity analysis of various antibodies was not extensively attempted. However, these MAbs did react with the amastigotes of strains AG83 and DD8 as well as with their respective infected cells. Taken together, this observation and that reported earlier for promastigotes (Goel, 1997), it appears that the epitopes recognized by these MAbs are cross-reactive among various stages of different strains of *L. donovani*. This cross-reactivity observed with cutaneous leishmaniasis causing species *L. major* promastigotes further revealed that the appearance of 44G3 and 33E8 reactive components was not a feature limited to RMRI strain and extends beyond the VL causing *L. donovani* to CL causing *L. major* and possibly to other *Leishmania* species.

Promastigote surface contains several antigens that are highly cross-reactive with other species of *Trypanosomatidae* and *Mycobacterium* (Smrkoviski and Gordon, 1977; Decker-Jackson and Honeigberg, 1978; Hedge 1978). However, the antigens recognized in this study are a unique feature of *Leishmania* species and no cross-reactive parallels were found in other intracellular pathogens *M. tuberculosis* and malaria parasite *P. berghei* (Table 2).

Thus, *Leishmania*-specific antigens (stage-specific or common to both parasite and infected cells) identified in this study might aid in: (i) understanding the mechanisms involved in the establishment of infection and, ii) developing better strategies for disease control.