Leishmaniases still constitute a major public health problem and the burden is increasing (Desjeux, 2001, Desjeux 2004b). In 2002, the World Health Organization (WHO) estimated the number of persons at risk to be around 350 million and the number of new cases to be 2357000 per year. In humans, the disease occurs in at least four major forms: cutaneous, diffuse cutaneous, mucocutaneous, and visceral (Desjeux, 2004a). Visceral leishmaniasis, also known as kala-azar, is the most severe form and is fatal if left untreated. In India it is endemic in eastern India, predominantly in Bihar State and West Bengal, where occasional epidemics occur. In the last two decades, sporadic cases have been reported from the eastern districts of Uttar Pradesh (Sundar and Chatterjee, 2006). Chemotherapy potentially leads to toxicity and other undesirable side effects and is becoming ineffective due to the emergence of drug resistance. In the absence of effective treatment, vaccination remains the only hope for control of the disease.

The present work was carried out to evaluate the protective efficacy of two heat shock proteins (Hsp70 and Hsp83) in combination with two different adjuvants (MPLA and ALD) in Leishmania donovani infected inbred BALB/c mice. The diagnostic potential of Hsp70 and Hsp83 was also evaluated using antigen detection method and western blotting.

Identification and electro-elution of Hsp70 and Hsp83

The parasite proteins were run on SDS-PAGE after staining and destaining the gel, the bands of interest were taken in the electrophoresis buffer in the electro-eluter. The proteins were eluted by applying constant voltage through the gel. After elution, the proteins were dialyzed, suspended in PBS and quantified.

Preparation of vaccines, Immunization and challenge infection of animals

Based on two heat shock proteins, Hsp70 and Hsp83, three types of vaccines were formulated with two adjuvants: autoclaved L. donovani antigen (ALD) and Monophosphoryl Monophosphoryl Lipid A (MPLA). The cocktail of these two heat shock proteins (without any adjuvant) was also used as a vaccine candidate for immunization. The Hsp70+Hsp83+ALD vaccine was formulated by mixing 250 μg of eluted proteins with 2.5 mg of ALD antigen while Hsp70+Hsp83+MPL-A vaccine was prepared by the addition of 100 μl solution of MPL-A (conc. 10 mg/ml) to 250 μg of the eluted proteins. BALB/c mice were immunized by subcutaneous injections of 10μg of Hsp70 and Hsp83 antigen free in PBS and along with different adjuvants.
Vaccine efficacy

The vaccine efficacy was assessed by analyzing parasite load in liver and spleen and generation of cell mediated and humoral immune responses.

Parasite load

Six animals from each group were sacrificed on 30, 60 and 90 days post infection/challenge. Liver and spleen of all animals were removed and weighed. Impression smears were made and the parasite load was assessed in terms of Leishman Donovan Units.

A significant reduction in parasite load was observed in all vaccinated groups on all post challenge days. Maximum parasite reduction was observed in Hsp70 + Hsp83 + MPLA vaccinated animals followed by Hsp70 + Hsp83 +ALD. Lowest protection was seen in animals vaccinated with the cocktail without any adjuvant.

The parasite load in spleen increased significantly from 30 to 60 days post infection and then declined significantly from 60 to 90 days post infection in the infected controls. However, it decreased significantly from 30 to 90 days post challenge in the vaccinated animals. The maximum splenic parasite load was seen in animals vaccinated with the cocktail of Hsp70 and Hsp83 without any adjuvant followed by Hsp70 + Hsp83 +ALD vaccinated group. Least parasite burden was observed in animals vaccinated with the Hsp70 + Hsp83 + MPLA.

Cell-mediated immune responses

DTH responses

The delayed type hypersensitivity responses (DTH) were measured by the percentage increase in footpad thickness after injecting leishmanin. The most pronounced DTH responses were observed in animals vaccinated with both the heat shock proteins plus MPLA followed by Hsp70 + Hsp83 +ALD and Hsp70 + Hsp83 vaccinated groups.

Cytokine responses

The immune responses augmented by three vaccines were analysed by quantifying the cytokines (IFN-γ, IL-2, IL-4 and IL-10) produced by spleen cells of immunized animals. Higher concentration of IFN-γ and IL-2 indicate protective Th1 type of immune response whereas higher concentration of IL-4 and IL-10 are indicators of non-protective Th2 type of immune response.

IFN-γ levels were significantly higher in the vaccinated groups as compared to the infected controls. The highest concentration of this cytokine was shown by the
animals vaccinated with the cocktail of Hsp83 and Hsp70 plus MPLA followed by the animals vaccinated with the two Hsps plus ALD. The lowest levels were revealed by the group immunized with only two Hsps without any adjuvant.

The patterns observed for IL-2 levels were similar to that observed for IFN-γ being higher in the vaccinated animals as compared to the infected controls. Maximum IL-2 levels were seen in the spleen cultures of mice vaccinated with two Hsps plus MPLA followed by the animals vaccinated with the two Hsps plus ALD. The animals immunized with only Hsps without any adjuvant revealed lowest concentration of this cytokine.

IL-4 levels were significantly lower in the vaccinated animals as compared to the infected controls. The concentration of this cytokine was minimal in mice vaccinated with the cocktail of Hsp83 and Hsp70+MPLA. Among immunized animals the highest concentrations of IL-4 were present in spleen cultures of mice vaccinated with only Hsp70+Hsp83.

The levels of IL-10 were also significantly lower in vaccinated animals as compared to infected controls. The least concentrations were observed in animals vaccinated with the Hsp83 and Hsp70 in combination with MPLA. The mice vaccinated with cocktail only revealed maximum concentration of IL-10 among the vaccinated animals.

**Humoral immune responses**

Humoral responses in vaccinated groups and controls were analysed by the distribution of IgG1 and IgG2a antibodies in their respective serum sample as detected by ELISA. The IgG1 which is an indicator of Th2 type of immune response, was found to be maximum in the infected controls as compared to the immunized animals. Among immunized groups minimal IgG1 response was seen in the group immunized with the cocktail of Hsp83 and Hsp70 in combination with MPLA. The mice vaccinated with cocktail only revealed maximum concentration of IL-10 among the vaccinated animals.

All the three vaccine formulations induced good IgG2a antibody response which is an indicator of protective Th1 type of immune response. However, maximum IgG2a antibody response was induced by Hsp83+Hsp70+MPLA followed by Hsp83+Hsp70+ALD and only Hsp83+Hsp70. Least antibody levels were observed in infected controls. The antibody levels increased from 30 to 90 days post infection/challenge in all groups of mice.
Summary and Conclusion

For diagnostic studies Hsp83 and Hsp70 were detected in serum samples of all the infected animals on 30, 60 and 90 d.p.i. A significantly higher absorbance was observed in infected animals as compared to normal un-infected animals on all days post infection. The absorbance was found to be higher during early infection. Further it declined on subsequent days post infection.

These two heat shock proteins were also recognized in serum samples of infected and immunized animals by western blotting. Both bands of Hsp70 and Hsp83 were simultaneously present in all infected controls as well as in immunized animals on all days post infection/challenge.

From these results, following conclusions can be drawn:

1. A considerable protective efficacy was shown by all vaccines against experimental murine leishmaniasis. It was evident from significant reduction in parasite load.
2. Significant DTH responses were generated in all vaccinated groups, the highest being in animals vaccinated with cocktail of Hsp83 and Hsp70 along with adjuvant MPLA. It suggests the generation of cell-mediated immune responses by these vaccines.
3. The higher levels of IgG2a, IFN-γ and IL-2 suggest the generation of protective Th1 type of immune responses in vaccinated groups.
4. The lower levels of IgG1, IL-4 and IL-10 suggest the abolishment of the non-protective Th2 type of immune responses.
5. The protective Th1 type of immune response was most pronounced in the group of animals vaccinated with two Hsps plus MPLA followed by the group vaccinated with two Hsps plus ALD. The lowest response was shown by the group of animals vaccinated with only cocktail of two Hsps.
6. The adjuvant MPLA was more effective than ALD.
7. Both antigens i.e. Hsp83 and Hsp70 have shown good diagnostic potential in experimental murine leishmaniasis.