To investigate the therapeutic potential of immunochemotherapy with cisplatin + 78 kDa + MPL-A against *Leishmania donovani* in BALB/c mice

J. JOSH & S. KAUR

Department of Zoology, Panjab University, Chandigarh, India

SUMMARY

Leishmaniasis has recently garnered attention as one of the diseases most neglected by drug research and development, as the current therapeutic modalities available for the patients are ridden with unacceptable toxicity due to high dosage of the drug, prolonged treatment schedules, resistance and prohibitive costs. A successful chemotherapy requires a restoration of immune response; therefore, we combined *Leishmania*-specific 78 kDa antigen (with or without adjuvant MPL-A) along with a novel drug cisplatin in infected BALB/c mice and did its comparative analysis with chemotherapy and immunotherapy alone. Animals that were treated with immunochemotherapy showed maximum curative potential as demonstrated by a marked reduction in parasite load. Delayed-type hypersensitivity response to leishmanial antigens has been widely used to assess the level of host protection to the disease. An increased delayed-type hypersensitivity (DTH) response was observed in animals given immunotherapy or chemotherapy or immunochemotherapy; however, maximum DTH response was observed in animals treated with cisplatin + 78 kDa + MPL-A. These animals were also found to exhibit higher IgG2a levels greater cytokine (IFN-γ and IL-2) concentrations suggesting the generation of a strong Th1 type of immune response which is responsible for resolution of the disease.

Keywords 78 kDa antigen, cisplatin, immunochemotherapy, MPL-A, visceral leishmaniasis

INTRODUCTION

Leishmaniasis a group of diseases caused by trypanosomatids from the genus *Leishmania*. The disease is found endemic in 88 countries all over the world, affecting 12 million people with an estimated 1.5-2.0 million new cases and 80,000 deaths each year (1). The disease is epidemiologically very diverse due to the large number of parasite species of genus *Leishmania* which are pathogenic to humans (2).

While substantial efforts have been made to develop vaccine-induced specific antiparasitic immune responses, no acceptable antileishmanial vaccine exists against this infection. The first vaccine to reach the human phase I clinical trials is Leish-F3 for visceral leishmaniasis (3), and Leishmune is the only licensed vaccine against the canine disease (4).

Glucose-regulated protein 78 (GRP78), also known as BiP (Binding protein), is a 78 kDa Ca⁺⁺ binding chaperone molecule and belongs to heat shock protein 70 family (HSP70). It has been observed that immunization of mice with 78 kDa antigen of *Leishmania donovani* increased the production of IgG2a titre and reduced spleen weight which correlated with the decrease in parasite load (5). Moreover, Nagill and Kaur (6) showed that 78 kDa in combination with various adjuvants imparts different degrees of protection in BALB/c mice against visceral leishmaniasis, maximum protection being observed in mice immunized with 78 kDa antigen in combination with rIL-12, MPL-A and liposome-encapsulated antigen.

In the absence of any suitable vaccine against visceral leishmaniasis (VL), chemotherapy is the only option for control of the disease. Although the treatment for leishmaniasis was introduced in the early 20th century, parenteral administration of pentavalent antimony compounds (meglumine antimoniate and sodium stibogluconate) remains the first-choice treatment for all forms of...
leishmaniasis (7). In the case of antimonial resistance, the second-choice treatment includes amphotericin B (dialcrolipol or liposomal formulation) (7). However, each of these therapies has important limitations, such as long-term parenteral administration, toxic side effects, high cost in endemic countries and an increase in number of resistance cases (8). A major breakthrough in chemotherapy of VL was the discovery of miltefosine, an analogue of phosphatidylcholine initially developed as an anticancer agent (9). It is not recommended during pregnancy as teratogenicity has been observed in one species during preclinical development. Moreover, its cost is another limiting factor (10). Till date, no ideal drugs are available that fulfill the major requirements for efficient antileishmanial therapy, including high efficacy, low toxicity, easy administration, low costs and avoiding occurrence of drug-resistant parasites (11).

Cisplatin (cis-diamminedichloroplatinum II; CDDP) is a platinum-based anticancerous drug, which mediates its action by forming cross-link of DNA ultimately triggering apoptosis, or programmed cell death (12), and is also known to enhance the cytotoxic immunity (13). An in vivo antileishmanial study with cisplatin at low dose also resulted in decreased parasite burden, increased delayed-type hypersensitivity (DTH) response, initial transient and reversible increase in various liver and kidney function tests (14). It is well known that nephrotoxicity is a dose-limiting factor of cisplatin, so later on, Sharma et al. (15) investigated the protective efficacy of high dose of cisplatin in combination with antioxidants (siilbinin, vitamin C and vitamin E) which effectively reversed the toxic side effects caused by the drug. So an auxiliary therapeutic measure that might enhance the efficacy of these antileishmanials or reduce the resulting toxicity would be valuable. Immuochemotherapy has been used with various combinations of drugs and vaccines mostly in case of cutaneous leishmaniasis. Some of them are sodium stibogluconate with poly ICLC (Polyinosinic-polycytidylic acid) plus arginine (16), antimony with interferon-gamma (17), N-methyl meglumine antimonate with recombinant Leish-110f plus MPL-SE vaccine (18), killed Leishmania promastigotes with antimonials (19) and alum precipitated autoclaved Leishmania promastigote (ALUM/ALM) plus BCG with sodium stibogluconate (20).

Chemotherapy of leishmaniasis is often compromised due to suppression of immune function during the course of infection. Therefore, efficiency of any chemotherapy of leishmaniasis is dependent on the generation of effective cell-mediated immune response suitable for disease resolution (21). Because of this close association between chemotherapy and cell-mediated immunity, treatment for L. donovani infection has been thought to be more amenable to combined therapy, that is, immunochemotherapy (16). Therefore, we tested immunochemotherapy to determine the safety, immunogenicity and probable curative potential of 78 kDa antigen in combination with a newly tested drug cisplatin in mice infected with L. donovani. The current study is expected to assist in the evaluation of immunochemotherapy as a better alternative antileishmanial therapy.

MATERIALS AND METHODS

Parasite and culture conditions

Promastigotes of L. donovani, strain MHOM/IN/80/Dd8, were grown at 22°C in NNN medium supplemented with MEM (pH 7.2), 200U of streptomycin, 200U of benzyl penicillin and 40 µg of gentamycin per mL and subcultured in the same medium after every 48–72 h.

Animals

Inbred BALB/c mice of either sex weighing 20–25 g were used for the present study. During the start of the experiment, the mice weigh around 20–25 g, but by the time, infection was given and treatment was completed weight increased to 25–30 g. These animals were obtained from Institute of Microbial Technology, Chandigarh, India, and then maintained in the Central Animal House, Panjab University, Chandigarh. All the mice were kept in appropriate cages and fed with water and food ad libitum throughout the study period. The ethical clearance for conducting various experiments on BALB/c mice was taken from Institutional Animal Ethics Committee (IAEC) of the Panjab University, Chandigarh.

Drug formulation

Cis-diamminedichloroplatinum (II) dichloride (CP) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) in the pure form, and then it was dissolved in distilled water to get the requisite concentration of 0.5 mg/kg body wt (14).

Identification and electro-elution of 78 kDa antigen

The 78 kDa antigen of L. donovani was identified and eluted as described by Nagill and Kaur (6).

Preparation of 78 kDa with MPL-A

The 78 kDa antigen alone (without any adjuvant) was also used as a vaccine candidate for immunization.
78 kDa + MPL-A vaccine was prepared by the addition of 144 \mu L solution of MPL-A (conc. 10 mg/mL) to 360 \mu g of 78 kDa antigen. Subcutaneous route was used for immunization of mice in all the groups (6).

**In vivo infection**

Mice were infected intracardially with $10^7$ promastigotes/0.1 mL (14).

**Treatment groups**

Animals were divided into different groups, and each group consisted of eighteen mice. Animals of Group 1 (Chemotherapy) received intraperitoneal injection of cisplatin at a dose of 0.5 mg/kg body wt. continuously for 5 days in two cycles with an interval of 14 days between each cycle, while Group 2 (cisplatin + 78 kDa) and Group 3 (cisplatin + 78 kDa + MPL-A) received immunochemotherapy, respectively. Animals in these groups were injected with cisplatin intraperitoneally in two cycles of 5 days with an interval of 14 days between each cycle, and two subcutaneous doses of vaccine were given at 18 days of interval, the first dose of vaccine was administered along with the first dose of drug treatment (as given in the flowchart). Animals in Group 4 and Group 5 received immunotherapy with 78 kDa and 78 kDa + MPL-A, respectively. This also consisted of two subcutaneous injections at same intervals. In Group 4, each mouse received 10 \mu g of 78 kDa, while in Group 5, each mouse received 10 \mu g of 78 kDa antigen along with 40 \mu g of MPL-A. Animals in Group 6 serve as positive controls (infected mice only) and in group 7 as negative controls (normal mice). Normal mice include those animals which were neither infected with promastigotes of *L. donovani* nor given any kind of treatment, whereas infected mice were given $1 \times 10^7$ promastigotes of *L. donovani* (Table 1).

**Parameters studied**

**Assessment of infection**

Six mice from each treated and control groups were euthanized on 1 (55 days post-infection (d.p.i.)), 15 (70 d.p.i.) and 30 (85 d.p.i.) post-treatment days (p.t.d.). Blood from different treated and control animals was collected by jugular vein incision. Then, blood was centrifuged to obtain serum, which was stored at -20°C until use. The liver and spleen of the individual animals were taken out and weighed. To quantitative levels of infection in liver and spleen, Giemsa-stained impression smears were made and fixed in methanol. The parasite load was assessed as Leishman-Donovan units (LDU) and calculated as: Number of amastigotes/Number of cell nuclei X weight of organ in milligrams (22).

**Delayed-type hypersensitivity responses**

Two days prior to the day of sacrifice, 20 \mu L (40 \mu g) of leishmanin was injected subcutaneously in right footpad and PBS in the left footpad of mice. After 48 h, the thickness of the both foot pads was measured using a pair of vernier callipers. The DTH response was evaluated in terms of percentage increase in footpad thickness according to the formula: difference between right and left footpad thickness/thickness of left footpad x 100 (23).

ELISA for parasite-specific IgG1 and IgG2a isotypes

Conventional ELISA was used to determine the levels of serum immunoglobulin G (IgG) isotype antibody (IgG1 and IgG2a) by the method of Kaur et al. (23). Shortly, 96-well plates were coated with 78 kDa antigen and incubated overnight at 4°C. After blocking with 4% bovine serum albumin, plates were incubated with serum samples at 37°C for 1 h followed by three washes and addition of 100 \mu L of anti-mouse secondary antibody conjugated with HRP in a dilution of 1 : 8400 of IgG1 (Serotec) and

**Treatment Schedule:**

- Infected mice (30 days infection)
- Immunochemotherapy
- Drug (5 days) + Vaccine (1 day)
- chemotherapy
- Drug (5 days) + Vaccine (1 day)
- Immunotherapy
- Drug (5 days) + Vaccine (1 day)
- Fourteen Days Rest
- Drug (5 days) + Vaccine (1 day)
- Mice were sacrificed on 1 (55 d.p.i.), 15 (70 d.p.i.), 30 (85 d.p.i.) days post treatment

© 2013 John Wiley & Sons Ltd, Parasite Immunology, 36, 3-12
Table 1 Groups of animals used in the study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Drug/vaccine dosages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chemotherapy</td>
<td>Cisplatin (0.5 mg/kg body wt.)</td>
</tr>
<tr>
<td>2</td>
<td>Immunochemotherapy</td>
<td>Cisplatin (0.5 mg/kg body wt.) + 78 kDa (10 µg)</td>
</tr>
<tr>
<td>3</td>
<td>Immunotherapy</td>
<td>Cisplatin (0.5 mg/kg body wt.) + 78 kDa (10 µg) + MPL-A (40 µg)</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>78 kDa (10 µg)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>78 kDa (10 µg) + MPL-A (40 µg)</td>
</tr>
<tr>
<td>6</td>
<td>Positive control</td>
<td>Infected only</td>
</tr>
<tr>
<td>7</td>
<td>Negative control</td>
<td>Normal</td>
</tr>
</tbody>
</table>

1: 2000 dilution of IgG2a (Serotec) and incubated further for 1 h at room temperature, after which the substrate and chromogen were added and absorbance read on ELISA reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Determination of cytokine responses

Lymphocytes from spleens of infected and drug-treated mice were seeded in 24-well plates in 1 mL of RPMI-1640 and incubated for 72 h at 37°C. Cells were stimulated with 50 µg/mL of the 78 kDa antigen. Supernatants of these cultures were collected and stored at −20°C. The release of cytokines (IL-2, IL-10, IL-4 and IFN-γ) was measured in the supernatants using commercial ELISA kits (Bender-Med Systems, Diaclone, France) (23).

Statistical analysis

All the data were analysed using two-way analysis of variance (ANOVA). Post hoc test was used for multiple comparisons using Holm-Sidak method. The results were considered statistically significant when \( P < 0.05 \).

RESULTS

Parasite load

The parasite burden in liver and spleen of mice was calculated in all groups of mice on 1, 15 and 30 post-treatment days and was measured in terms of LDU. Parasite load in liver increased significantly in infected control BALB/c mice on different post-infection days. In contrast, in the treated animals, the parasite load declined significantly (\( P < 0.05 \)) from 1 to 30 post-treatment days. Among the three treatments, that is, chemotherapy, immunotherapy and immunochemotherapy, the last was the most effective in reducing the parasite load. Cisplatin treatment reduced the hepatic parasite load of mice by 63.06%, 68.37% and 72.50% on 1, 15 and 30 p.t.d., respectively. Addition of 78 kDa to these drugs further declined the parasite load significantly. The LDU declined by 75.95-83.95% as compared to the infected controls from 1 to 30 p.t.d. (Figure 1a). Moreover, addition of MPL-A further lessened the parasite load by 84.38-93.23% as compared to the infected controls from 1 to 30 p.t.d. The splenic parasite burden was also significantly reduced in all the treated groups as compared to control animals (Figure 1b).

Delayed-type hypersensitivity responses

The DTH responses increased significantly (\( P < 0.05 \)) from 1, 15 to 30 days post-treatment in all groups of animals. The treated animals revealed significantly (\( P < 0.05 \)) higher DTH responses in comparison with the infected controls. However, the animals treated with immunochemotherapy revealed significantly higher DTH responses compared with chemotherapy alone or immunotherapy alone. Treatment of animals with cisplatin + 78 kDa + MPL-A induced the highest DTH responses followed by cisplatin + 78 kDa and then cisplatin. Individual treatments generated significantly lesser DTH responses in comparison with those given in combination. (Figure 2).

Specific antibody responses

IgG1 and IgG2a antibody responses were also evaluated by ELISA using specific anti-mouse isotype antibodies in the sera of treated and control animals. Treated animals showed higher IgG2a and lower IgG1 antibody levels in comparison with the infected controls. Absorbance levels of IgG2a were maximum in animals treated with immunochemotherapy. Heightened antibody response was observed in cisplatin + 78 kDa + MPL-A-treated animals followed by cisplatin + 78 kDa and then cisplatin. Individual treatments generated significantly lesser DTH responses in comparison with those given in combination. (Figure 3a).

In contrast to the IgG2a levels, the treated animals revealed significantly (\( P < 0.05 \)) lesser IgG1 levels as compared to the infected controls. Immunochemotherapy-treated groups produced lesser IgG1 response as compared to chemotherapy or immunotherapy alone (\( P > 0.05 \)). The animals treated with cisplatin in combination with 78 kDa...
alone or with adjuvant MPL-A produced lesser IgG1 levels as compared to those treated with 78 kDa alone or 78 kDa + MPL-A ($P > 0.05$). Minimum IgG1 levels were observed in the animals immunized with cisplatin + 78 kDa + MPL-A (Figure 3b).

Cytokine responses

The concentration of Th1-specific cytokines, that is, IFN-γ and IL-2, was significantly ($P < 0.05$) higher in the treated mice as compared to the infected controls. Maximum concentrations of these cytokines were observed in animals treated with the combination of cisplatin + 78 kDa along with MPL-A. As compared to this group, the mice immunized with cisplatin + 78 kDa showed significantly ($P < 0.05$) lesser concentration of these cytokines. Least concentration of these cytokines was observed in the animals treated with the immunotherapy alone (Figure 4a,b). The levels of Th2-regulated cytokine, IL-10 and IL-4, were significantly lesser in treated animals as compared to the infected controls. Maximum levels of this cytokine were observed in the infected controls. Animals treated with cisplatin + 78 kDa + MPL-A showed least concentration of IL-10 and IL-4 (Figure 5a,b). As compared to this group, the concentration of the cytokine was significantly ($P < 0.05$) higher in the animals treated with cisplatin + 78 kDa followed by cisplatin.

© 2013 John Wiley & Sons Ltd, Parasite Immunology, 36, 3–12
Figure 2: Percentage increase in footpad thickness (delayed-type hypersensitivity (DTH) response) in infected and drug-treated mice on different post-treatment days. The data are presented as mean ± SD of six mice per group. (#) P value: Infected only vs. Infected + 78 kDa vs. Infected + 78 kDa-MPL-A; (*) P value: Infected only vs. Infected + cisplatin vs. Infected + cisplatin + 78 kDa vs. Infected + cisplatin + 78 kDa + MPL-A. (#), (*) — (P < 0.05).

Figure 3: Levels of Leishmania-specific IgG antibodies in serum samples of BALB/c mice. (a) IgG2a. (b) IgG1. The data are presented as mean ± SD of six mice per group. (#) P value: Infected only vs. Infected + 78 kDa vs. Infected + 78 kDa-MPL-A; (*) P value: Infected only vs. Infected + cisplatin vs. Infected + cisplatin + 78 kDa vs. Infected + cisplatin + 78 kDa + MPL-A. (#), (*) — (P < 0.05).
DISCUSSION

It has been well established that the success of any chemotherapy is often dependent on the type of immune response generated by the infected host, and in leishmaniasis, a drug is considered successful if it results in generation of antigen-specific T cells and delayed hypersensitivity. Due to the existence of close association between chemotherapy and cell-mediated immunity, immunochemotherapy is thought to be more agreeable for treatment for VL. Therefore, in the present study, the therapeutic potential of immunochemotherapy was tested by treating the BALB/c mice with a novel antileishmanial drug cisplatin along with a 78 kDa antigen formulated with an adjuvant (MPL-A).

Earlier studies have shown that significant inhibition was observed in golden hamsters infected with *L. donovani* when treated with a combination of low doses of both Stibanate and poly ICLC plus L-arginine (16). Similarly, we tested a low dose of cisplatin (0.5 mg/kg body wt.) and combined it with 78 kDa antigen along with the adjuvant as parasite antigens that preferentially stimulate the induction of significant protection through Th1 response represents a rational approach for vaccines against leishmaniasis. This has been demonstrated in our earlier study carried out by Nagill and Kaur, (6) where experimental infection of mice immunized with 78 kDa antigen along with MPL-A induced significant protection against *L. donovani* infection. Maximum reduction in parasite load in the present study was observed in animals treated with cisplatin + 78 kDa + MPL-A followed by cisplatin + 78 kDa more than any individual therapy. This is in consistence to an earlier study carried out by Tan et al., (24) which showed that both low-dose cisplatin (0.6 mg/ kg) and xenogenic endoglin (10 μg/mouse) resulted in significant tumour growth inhibition. During the course of
this treatment, promotion of tumour cell apoptosis and inhibition of tumour cell proliferation without any increase in host toxicity were also observed. Moreover, combination therapy using cisplatin and human leucocyte antigen-A24-restricted human vascular endothelial growth factor receptor 1 (VEGFR1)-1084 and VEGFR2-169 in patients with advanced or recurrent adenocarcinoma of the stomach showed that the disease control rate (partial and stable disease) was 100% after two cycles of the combination therapy (25).

Delayed-type hypersensitivity response to leishmanial antigens has been widely used to assess the level of host protection to the disease (26). It has been well established that induction of a DTH response is mediated via Th1 cell as it secretes IFN-\(\gamma\) which is expressed during macrophage stimulation for parasite killing (27). The DTH responses to leishmanin were apparent during \(L.\) \textit{donovani} infection in BALB/c mice as evident by an increase in the foot pad swelling after injection of leishmanin. The increase was much higher when the animals were treated with immunochemo-therapy than the groups of animals treated with chemotherapy or immunotherapy alone. This suggests that the mice treated with cisplatin + 78 kDa with or without adjuvant (MPL-A) developed a strong cell-mediated immune response indicating that drug treatment followed by vaccine therapy was helpful in reversal of immunosuppression caused by the parasite. Earlier studies from our laboratory reported an increased DTH response.
in animals treated with low dose of cisplatin (14). Correlation between DTH responses and parasite load has also been reported (14, 15). This was evident from our results where a strong positive correlation was observed between enhanced DTH response and reduced parasite load.

The immunological response was further characterized by analysing the distribution of IgG1 and IgG2a specific antibodies in the serum samples of infected and treated BALB/c mice. Production of IgG2a is normally associated with IFN-γ secretion and the development of a Th1 immune response. However, in contrast, production of the IgG1 is normally associated with IL-4 secretion and the development of Th2 type of response. The treated animals revealed higher IgG2a and lower IgG1 levels than the infected controls. However, maximum levels of IgG2a and minimum levels of IgG1 were observed in animals treated with cisplatin + 78 kDa + MPL-A than those animals that are treated with cisplatin alone or 78 kDa/78 kDa + MPL-A alone. It has been shown earlier from our laboratory that immunization of mice with 78 kDa + MPL-A resulted in significant increase in IgG2a response (6). Moreover, a significant reduction in specific antibody titres was observed after treatment with immunochemotherapy (Glucantime + Leish-11017/MPL-SE) in dogs suffering from canine leishmaniasis (18).

Th1 and Th2 cell lymphocytes are important mediators in generating immunity to leishmaniasis and can be distinguished by the cytokines they secrete. It may cause the secretion of IFN-γ and TNF-α, which promotes macrophage activation and thus contribute to disease resolution, or to the secretion of IL-10 or TGF-β associated with macrophage deactivation and inhibition of IFN-γ thus promoting the exacerbation of disease (28). In our study, high levels of cytokines were observed in all the animals after treatment. This has been shown earlier that patients with kala-azar usually show expansion of parasite-specific lymphocytes, and long-term T-cell responses are maintained even after clinical cure (29). However, compared with chemotherapy, immunotherapy and immunochemotherapy, maximum absorbance in Th1 cytokine levels (IFN-γ and IL-2) and minimum levels of Th2 cytokines (IL-10, IL-4) were observed in animals treated with immunochemotherapy. Moreover, maximum levels of Th1 cytokines and minimum levels of Th2 cytokines were produced by cisplatin + 78 kDa + MPL-A. This is in accordance to a study which stated that restoration of cell-mediated immunity to the parasite is necessary for an effective pentavalent antimonial therapy (30). Our results are in correspondence to a study carried out by Miss et al., (20) who observed that the healing process in PKDL patients was due to modulation of patients immune system tipping the Th1/Th2 immune response to a pure Th1 response. Moreover, the dogs that were given immunotherapy showed a significantly increased percentage of T helper lymphocytes, that is, the percentage of CD4/TcRβ+ and CD4/CD45RA+ cells increased significantly which are associated with disease remission (31).

To conclude, the present study puts an insight into the use of immunochemotherapy with a combination of drug and vaccine formulation. As the standard antileishmanials used to treat leishmaniasis are met with various side effects: therefore, low dose of cisplatin in combination with L. donovani specific 78 kDa antigen along with adjuvant MPL-A can prove to be a good alternative for the treatment for visceral leishmaniasis. However, more studies are required to test the combination in higher animal models before it is tested in VL patients.

ACKNOWLEDGEMENTS
The authors acknowledge the support provided by the PURSE Grant of Department of Science and Technology, and University Grant Commission, Fellowship programme, India.

COMPETING INTERESTS
The authors have no competing interests.

AUTHORS CONTRIBUTION
Both the authors have materially participated in the research work and article preparation. Jyoti Joshi and Sukhbir Kaur conceived and designed the experiments. JJ performed the experiments and helped by SK to analyse the data. SK contributed reagents/materials for the experiment. JJ wrote the paper. SK gave necessary suggestions and finally approved the manuscript to be submitted for publication.

REFERENCES
Studies on the protective efficacy of second-generation vaccine along with standard antileishmanial drug in *Leishmania donovani* infected BALB/c mice

**JYOTI JOSHI and SUKBIR KAUR***

Department of Zoology, Panjab University, Chandigarh 160014, India

(Received 7 August 2013; revised 27 September 2013; accepted 14 October 2013)

**SUMMARY**

It is well established that visceral leishmaniasis (VL; also known as Kala azar) causes immunosuppression, and a successful drug treatment is associated with the development of cell-mediated immunity. Therefore combining a drug with an immune enhancer can provide a better approach for the treatment of the disease. Keeping this in mind, the *in vivo* antileishmanial efficacy of immunochemotherapy was evaluated with the use of a 78 kDa antigen with or without monophosphoryl lipid A (MPL-A) along with a traditional drug sodium stibogluconate (SSG) in *Leishmania donovani* infected BALB/c mice. Mice were infected intracardially with promastigotes of *L. donovani*, and 30 days after infection, these animals were given specific immunotherapy (78 kDa/78 kDa + MPL-A) or chemotherapy (SSG) or immunochemotherapy (SSG + 78kDa/SSG+ 78 kDa + MPL-A). Animals were euthanased on 1, 15 and 30 post-treatment days. The antileishmanial potential of the immunochemotherapy was revealed by significant reduction in the parasite burden (*P*<0.001). These animals were also found to exhibit increased delayed type hypersensitivity (DTH) responses, higher IgG2a levels, lower IgG1 levels and greater cytokine (IFN-γ and IL-2) concentrations compared with chemotherapy or immunotherapy alone, pointing towards the generation of a strong protective (Th1) type of immune response. Immunochemotherapy with SSG + 78 kDa + MPL-A was found to be most effective in protecting mice against VL and therefore can be an alternative option for treatment of VL.

**Key words:** Visceral leishmaniasis, immunochemotherapy, sodium stibogluconate, 78 kDa antigen, MPL-A.

**INTRODUCTION**

Visceral leishmaniasis (VL) is ranked second in mortality and fourth in morbidity among tropical diseases, with 20 000 to 40 000 deaths per year and over 2 million DALYs (disability-adjusted life years) lost (McCall *et al.* 2013). Over the past decades, alternative drugs or new formulations of old ones have become available but, as yet, none of them are ideal for treatment due to high toxicity, resistance issues, prohibitive prices, long treatment length or inadequate mode of administration not adapted to the field. In addition, many patients are unable to complete the whole treatment, increasing the risk of drug resistance development (Freitas-Junior *et al.* 2012).

Following the observation that recovery from infection confers immunity to reinfection in leishmaniasis, the development of effective vaccines represents one of the most promising approaches for providing cost-effective interventions against this disease. The first vaccine against leishmaniasis, popularly known as leishmanization, was developed in the early 1940s. Due to its hazardous effects, leishmanization was replaced by first-generation vaccines, composed of killed parasites or crude extracts and live attenuated parasites (Modabber, 2010). However, the success of these vaccines in humans has generally been poor due to the failure to elicit adequate cellular immunity, an essential feature for the control of intracellular infections (Roberts, 2006). The newer vaccines under consideration comprise recombinant DNA-derived antigens and peptides. Some of the target antigens are species and life-cycle stage specific, while others are shared by promastigotes and amastigotes (Handman, 2001). These second and the third-generation sub-unit vaccines include gp63, LACK, LeIF, TSA, 78 kDa antigen etc. (Nagill and Kaur, 2011). Glucose-regulated protein 78 (GRP78), also known as BiP (Binding protein) is a 78 kDa Ca\(^{2+}\) binding chaperone molecule and belongs to the heat shock protein 70 family (HSP70). Nagill and Kaur (2011) showed that immunization with 78 kDa in combination with various adjuvants imparts different degrees of protection in BALB/c mice against VL, maximum protection being observed in mice immunized with 78 kDa antigen in combination with rIL-12, MPL-A and liposome encapsulated antigen.

Immunotherapy with or without chemotherapy has been used for treatment of cutaneous leishmaniasis in Venezuela for more than a decade. In Brazil, repeated daily doses of killed *Leishmania amazonensis*
vaccine (‘Mayrink vaccine’) (Mayrink et al. 1992) are given with a low dose of antimonial (8 mg kg⁻¹ body weight per day) for four cycles of 10 injections given daily, followed by a 10-day rest. The vaccine has been registered in Brazil as an adjunct for low-dose chemotherapy. In Sudan, a trial involving patients with persistent PKDL showed that the cure rate with immunotherapy was significantly higher than that with chemotherapy alone (sodium stibogluconate; SSG) (final cure rates: 87 and 53%) respectively. The vaccine was a mixture of killed Leishmania major adsorbed on alum plus BCG, given four times at weekly intervals (Musa et al. 2008).

While there may be a therapeutic role for immunomodulators in the future, none is available or recommended for routine use at present. As therapeutic vaccines can be evaluated rapidly at lower cost, immunochemotherapeutic vaccines may be achievable. Therefore, we tested immunotherapy to determine the safety, immunogenicity and probable curative potentials of 78 kDa antigen in combination with a conventional drug (SSG) in mice infected with Leishmania donovani. The current study is expected to assist in the evaluation of immunochemotherapy as an improved antileishmanial therapy.

MATERIALS AND METHODS

Parasite and culture conditions

Leishmania donovani promastigotes of strain MHOM/IN/80/Dd8, originally obtained from the London School of Hygiene and Tropical Medicine, London, were used for the present study and maintained in vitro at 22 ± 1 °C in modified NNN medium by serial cultures after every 48—72 h (Rao et al. 1984).

Animals

Inbred BALB/c mice of either sex, weighing 20–25 g were used for the present study. The animals were obtained from the Institute of Microbial Technology, Chandigarh, India and then maintained in the Central Animal House, Panjab University, Chandigarh. They were kept in appropriate cages with water and food ad libitum throughout the experiment time. The ethical clearance for conducting various experiments mentioned in the study on BALB/c mice was obtained from the Institutional Animal Ethics Committee (IAEC) of the Panjab University, Chandigarh, India.

Drug formulations

SSG was obtained from Wellcome Research Laboratories, UK in the pure form and dissolved in distilled water at 72 °C to get a concentration of 40 mg kg⁻¹ body wt (Sodhi et al. 1992).

Identification and electroatraction of 78 kDa antigen

The 78 kDa antigen of L. donovani was identified by 2-dimensional gel electrophoresis with the help of molecular weight markers as described by Nagill and Kaur (2010).

Preparation of 78 kDa with MPL-A

The 78 kDa antigen alone (without any adjuvant) was also used as a vaccine candidate for immunization. 78 kDa + MPL-A vaccine was prepared by the addition of 144 µL solution of MPL-A (conc. 10 mg mL⁻¹, and purchased from Sigma Aldrich Co., USA) to 360 µg of 78 kDa antigen. Subcutaneous route was used for immunization of mice in all the groups (Nagill and Kaur, 2010).

Experimental infection

In vivo infection: Mice were infected intraperitoneally with 10⁵ promastigotes/0.1 mL (Kaur et al. 2010).

Treatment groups

Animals were divided into different groups. Each group consisted of 18 mice as shown in Table 1.

Group 1 (Chemotherapy): Mice from each group received intraperitoneal injection of SSG (40 mg kg⁻¹ body wt) for 2 cycles of 5 injections daily followed by 14 days rest.

Group 2–3 (Immunotherapy): Animals in these groups were given a combination of drug and vaccine i.e. Group 2 animals were given SSG + 78 kDa and animals in Group 4 were injected with SSG + 78 kDa + MPL-A. In both of these groups, SSG was injected intraperitoneally in 2 cycles of 5 doses each with an interval of 14 days between each cycle and 2 subcutaneous doses of vaccine were given at 18 days interval; the first dose of vaccine was administered along with the first dose of drug treatment.

Group 4–5 (Immunotherapy): Treatment of animals that received immunotherapy only, that is 78 kDa alone (Group 4) and 78 kDa + MPL-A (Group 5), also consisted of 2 subcutaneous injections at same intervals. In Group 4, each mouse received 10 µg of 78 kDa antigen while in Group 5, each mouse received 10 µg of 78 kDa antigen along with 40 µg of MPL-A.

Group 6 served as positive control (Infected mice only) and Group 7 served as negative control (naive mice). Naive mice includes those animals which were neither infected with promastigotes of L. donovani nor given any kind of treatment whereas Infected mice were given 1 x 10⁵ promastigotes of L. donovani.

Parameters studied

Assessment of infection. Six mice from each group were euthanased on 1 (55 days post infection (dpi),...
Table 1. Various groups used in the study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Drug/vaccine dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chemotherapy</td>
<td>SSG (40 mg kg⁻¹ body wt)</td>
</tr>
<tr>
<td>2</td>
<td>Immmunochemotherapy</td>
<td>SSG (40 mg kg⁻¹ body wt) + 78 kDa antigen (10 µg)</td>
</tr>
<tr>
<td>3</td>
<td>SSG (40 mg kg⁻¹ body wt) + 78 kDa antigen (10 µg) + MPL-A (40 µg)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Immunotherapy</td>
<td>78 kDa antigen (10 µg)</td>
</tr>
<tr>
<td>5</td>
<td>78 kDa antigen (10 µg) + MPL-A (40 µg)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Positive control</td>
<td>Infected only</td>
</tr>
<tr>
<td>7</td>
<td>Negative control</td>
<td>Naive mice</td>
</tr>
</tbody>
</table>

15 (70 dpi) and 30 (85 dpi), post treatment days (ptd). Livers and spleen of all the animals were aseptically removed and their impression smears were microscopically examined after fixing and staining the slides with Giemsa. In order to quantitate levels of infection, Leishman Donovan units (LDU) were calculated as: Number of amastigotes/Number of cell nuclei x weight of organ in milligrams (Bradley and Kirkley, 1977).

Delayed type hypersensitivity (DTH) responses. All groups of mice were challenged in the right foot pad with a subcutaneous injection of leishmanin in the right foot pad and left foot pad with PBS. For preparing leishmanin, promastigotes in the stationary phase of growth were harvested from modified NNN medium and washed thrice with PBS (phosphate buffer saline). The final pellet was then suspended in 5 mL of 0.5% phenol in sterile PBS and kept at room temperature for 10 min. The phenol was then removed and the final concentration was adjusted to 2 x 10⁶ promastigotes per mL.] After 48 h, the thickness of the right and left foot pads was measured by using a pair of vernier callipers. The percentage increase in the thickness of the right foot pad as compared with the left was calculated (Kaur et al., 2008).

ELISA for parasite-specific IgG1 and IgG2a isotypes. The specific serum immunoglobulin G (IgG) isotype antibody (IgG1 and IgG2a) response was measured by conventional ELISA following the method of Kaur et al. (2008).

Determination of cytokine responses. The lymphocytes from spleens of infected and drug-treated mice were cultured in 24-well plates in 1 mL of RPMI-1640 containing 20 mM NaHCO₃, 10 mM HEPES, 10 U/mL streptomycin and 2 mM L-glutamine and 10% FCS. Cells were stimulated with 50 µg mL⁻¹ of the respective antigen (78 kDa or crude antigen) and then cells were incubated at 37°C for 72 h and cell free supernatants were collected and stored at −20°C. This was then assayed for IL-2, IL-10, IL-4 and IFN-γ by using ELISA kits (BenderMed Systems, Diaclone, France) (Kaur et al. 2008).

Statistical analysis. Two-way analysis of variance (ANOVA) and Post-hoc tests were used to investigate differences between groups. All Pairwise Multiple Comparison Procedures were performed using the Holm-Sidak method. The results were considered statistically significant when P<0.001 and P<0.05.

RESULTS

Parasite load

The parasite burden in liver and spleen of mice was calculated in all groups of mice on 1, 15 and 30 ptd and was measured in terms of LDU. Parasite load in liver increased significantly in infected control BALB/c mice on different post infection days. In contrast, in the treated animals, the parasite load declined significantly (P<0.05) from 1 to 30 ptd. In the animals treated with SSG, the parasite load declined by 88.49 to 91.27% as compared with the infected controls from 1 to 30 ptd. When the animals were given immunochemotherapy and treated with SSG along with 78 kDa antigen alone or with adjuvant MPL-A, the level of protection increased significantly (P<0.001). Animals treated with SSG+78 kDa showed a reduction of 90.97–97.80% in the parasite load while least parasite load was observed in animals treated with SSG + 78 kDa in combination with MPL-A. The decline in parasite load in this group of animals was 95.34–99.44% as compared with the infected controls (Fig. 1). The splenic parasite burden was also significantly reduced in all the treated groups as compared with control animals (data not shown).

Delayed type hypersensitivity responses

The DTH responses were measured as an index of cell-mediated immune responses. In all the groups of animals, the DTH responses increased significantly from 1 to 30 ptd. The treated animals revealed significantly (P<0.001) higher DTH responses in comparison to the infected controls. However, animals treated with SSG+78 kDa along with MPL-A induced the highest level of DTH response followed by those treated with SSG+78 kDa and SSG (Fig. 2).
Fig. 1. Parasite load in terms of LDU in infected and drug treated mice on different post treatment days. The data are presented as mean±s.D. of six mice per group. (*) P value: Infected only vs Infected +78 kDa/Infected +78 kDa-MPL-A/Infected +SSG/Infected +SSG +78 kDa/Infected +SSG +78 kDa-MPL-A; (l) P value: Infected +SSG vs Infected +SSG +78 kDa/Infected +SSG +78 kDa-MPL-A; (a) P value: Infected +78 kDa vs Infected +SSG +78 kDa-MPL-A. (*), (l), (a) -(P<0.001); (g) -(P<0.05).

Fig. 2. Percentage increase in footpad thickness (DTH response) in infected and drug-treated mice on different post treatment days. The data are presented as mean±s.D. of six mice per group. (*) P value: Infected only vs Infected +78 kDa/Infected +78 kDa-MPL-A/Infected +SSG/Infected +SSG +78 kDa/Infected +SSG +78 kDa-MPL-A; (l) P value: Infected +SSG vs Infected +SSG +78 kDa/Infected +SSG +78 kDa-MPL-A; (a) P value: Infected +78 kDa vs Infected +SSG +78 kDa-MPL-A. (*), (l), (a) -(P<0.001).

Specific antibody responses

The humoral response was characterized by analysing the distribution of IgG1 and IgG2a specific antibodies in the sera of treated and control animals. In all the groups of animals, IgG1 and IgG2a antibody responses were evaluated in the serum samples by ELISA using specific anti-mouse isotype antibodies. Treated animals showed higher IgG2a and lower IgG1 antibody levels in comparison to the infected controls (P<0.001). The maximum levels of IgG2a antibody were observed in the mice treated
with SSG + 78 kDa + MPL-A $(P<0.001)$. The SSG + 78 kDa recipient mice also produced significant levels of IgG2a antibody (Fig. 1A). In contrast to the IgG2a levels, the treated animals revealed significantly $(P<0.001)$ lesser IgG1 levels as compared with the infected controls. The animals treated with SSG in combination with 78 kDa alone or with adjuvant MPL-A revealed significantly $(P<0.05)$ reduced IgG1 levels as compared with those treated with 78 kDa alone or 78 kDa + MPL-A. Minimum IgG1 levels were observed in the animals immunized with SSG + 78 kDa + MPL-A (Fig. 3B).

**Cytokine responses**

The concentration of Th1 specific cytokines, that is, IFN-γ and IL-2 were significantly $(P<0.001)$ higher in the treated mice as compared with the infected controls. Maximum concentrations of these cytokines were observed in animals treated with the combination of SSG + 78 kDa along with MPL-A. As compared with this group, the mice immunized with SSG + 78 kDa showed significantly $(P<0.05)$ lesser concentration of these cytokines. Least concentration of these cytokines was observed in the animals treated with the immunotherapy alone (Fig. 4A and B). The levels of Th2 regulated cytokine IL-10 $(P<0.001)$ and IL-4 $(P<0.05)$ were significantly lesser in treated animals as compared with the infected controls. Maximum levels of this cytokine were observed in the infected controls. Animals treated with SSG + 78 kDa + MPL-A showed least concentration of IL-10 and IL-4 (Fig. 5A and B) $(P<0.001)$. As compared with this group, the concentration of the cytokine was significantly $(P<0.05)$
Fig. 4. Effect of treatment on 

A–IFN-γ, B–IL-2 cytokine levels of BALB/c mice. The data are presented as mean±s.D. of six mice per group. (*, #) P value: Infected only vs Infected +78 kDa/Infected +78 kDa + MPL-A; !, @) P value: Infected + SSG vs Infected + SSG + 78 kDa/Infected + SSG + 78 kDa + MPL-A; (a) P value: Infected + 78 kDa vs Infected + SSG + 78 kDa + MPL-A. (*), (!), («) (P<0.001); (#), (@) (P<0.05).

higher in the animals treated with SSG + 78 kDa followed by SSG.

DISCUSSION

For the control of leishmaniasis, although improved drug regimens and new drug combinations are becoming available, complete elimination of the parasite is never achieved. Similarly, a combination of drugs and vaccines or even vaccine alone has been successfully used to treat American cutaneous leishmaniasis over the past years (Mayrink et al. 2006), with a low number of recurrence cases. Combined anti-Leishmania treatment using vaccine and drugs would enhance drug efficacy and stimulate host immune responses, resulting in a therapeutic harness of cellular immune protection (Borja-Cabrera et al. 2010). Parasite antigens that preferentially stimulate the induction of significant protection through Th1 response present a rational approach for a vaccine against leishmaniasis. Therefore, in the present study the therapeutic potential of immunochemotherapy was tested by treating infected mice with a pentavalent antimony drug (SSG) along with 78 kDa antigen formulated with an adjuvant (MPL-A).

To assess the protective efficacy of immunochemotherapy against murine VL, the parasite load was analysed in terms of LDU. Maximum killing of the parasite was observed in animals treated with a combination of a drug and a second-generation vaccine. About 99.49% protection was observed which suggested more effectiveness of the drug with a specific antigen along with an adjuvant. These results are consistent with our earlier studies where experimental infection of mice immunized with second-generation antigen (78 kDa) along with an adjuvant (MPL-A) induced 92% protection against L. donovani infection (Nagill and Kaur, 2010). Similarly,
Fig. 5. Effect of treatment on A -IL-10, B -IL-4 cytokine levels of BALB/c mice. The data are presented as mean±s.d. of six mice per group. (*) P value: Infected only vs Infected+78 kDa/Infected+SSG/Infected+SSG+78 kDa/Infected+SSG+78 kDa+MPL-A; (l) P value: Infected+SSG vs Infected+SSG+78 kDa/Infected+SSG+78 kDa+MPL-A; (a) P value: Infected + 78 kDa vs Infected + SSG + 78 kDa + MPL-A. (*) (l), (a) - (P<0.001); (l) - (P<0.05).

in an earlier study, the therapeutic efficacy of Leishmune, the only licensed vaccine against canine leishmaniasis, was assessed for immunochemotherapy in combination with allopurinol or amphotericin B in dogs. It was observed that by the end of 8 months, no parasite antigens were observed in lymph nodes of 80% of immunochemotherapy-treated dogs. This suggests that the combination therapy not only abolished the symptoms but also the latent infection, curing the dogs (Borja-Cabrera et al. 2010). Moreover, Murray et al. (2003) tested an immunochemotherapy protocol in L. donovani-infected mice by the association of amphotericin B with IL-12 and observed that, despite this drug’s direct action against parasites and its independence from host immunity, the combination was more efficient than the monotherapy. In our study, SSG + 78 kDa + MPL-A has shown maximum elimination of parasites because of the combination of SSG + 78 kDa with adjuvant MPL-A. Several studies have demonstrated that the adjuvant either directly or indirectly stimulates the production of the T helper cell type 1 (Th1) cytokines IL-2 and IFN-γ (Gustafson and Rhodes, 1994). In addition, MPL-A activates monocytes and macrophages (Ribi et al. 1984). It is likely that these monokines lead to the recruitment and maturation of dendritic cells in the lymph nodes (Jonuleit et al. 1996) where the dendritic cells can efficiently present antigen to T lymphocytes. Therefore, the combination has been found to be most effective in eliminating the parasites. However, it was reported earlier that complete clearance of parasites did not occur even from liver when BALB/c mice infected with high dose of L. donovani parasites were treated with anti-IL-10 Ab in combination with pentavalent antimonials or AmB (Murray et al. 2003; Banerjee et al. 2008). It has been reported that in genetically susceptible
(Nraamp10) mice (including BALB/c and C57BL/6), parasite numbers rapidly expand in hepatic mononuclear phagocytes, followed over the subsequent 2–4 weeks post-infection by a T cell-dependent decline in tissue parasite numbers (Bradley and Kirkley, 1977) whereas parasite numbers increase more slowly in the spleen, and total splenic parasite burdens usually only reach 5–10% of maximum levels in the liver, often with greater variation between individual mice than in the livers of the same animals (Stanley et al. 2008). Moreover, as BALB/c mice develop self-limiting infection, no relapse occurs in them. Hence, it was assumed that neither chemotherapy nor satisfactorily expressed T cell-dependent host immune responses could eradicate all tissue parasites in any form of leishmaniasis (Banerjee et al. 2008).

Delayed-type hypersensitivity (DTH) is the cell-mediated immune response for the clearance of pathogen from infected host that potentiates the infiltration of lymphocytes and macrophages into the infected tissue (Khabiri et al. 2007) and has been frequently used as a correlate for protection against or sensitization to Leishmania antigen (Hamid et al. 2007). The DTH responses to leishmanin after treatment were much higher in mice treated with a combination of drug and an immunomodulator than the groups of animals treated with chemotherapy or immunotherapy alone. The DTH responses are also evaluated in correlation with parasite burden. The higher the DTH responses, the lesser is the parasite load and greater is the efficacy of the drug (Sharma et al. 2012). Our results also demonstrate a negative correlation between enhanced DTH responses and reduced parasite load for all the therapeutic treatments.

We further characterized the immunological response by analysing the distribution of IgG1 and IgG2a specific antibodies in the serum samples of BALB/c mice. The serum samples of treated animals showed higher antibody titres with elevated levels of Th1 cytokine-regulated antibody, IgG2a when compared with infected mice. IgG2a titres were highest in mice that were treated with a combination of a drug and a second-generation antigen.

It has been well established that profound impairment of the immune system of the infected host in VL is a major cause for partial success of the anti-leishmanial chemotherapy, and success of cure depends on the combined effect of drug and immune status of the host (Banerjee et al. 2008). Recovery from leishmaniasis is strongly co-related with the development of distinct T helper type 1 (Th1) cell mediated immune responses manifested by the production of cytokines such as IFN-γ and IL-2 by T cells that activate macrophages and kill intracellular parasites (Dey et al. 2013). In contrast, Th2 cell-mediated immune response limits the action of Th1 functions via IL-10 which deactivate macrophages helping intracellular parasite growth and disease progression (Awasthi et al. 2004). IL-10 has been suggested to play a role in counterbalancing the exacerbated polarized response that may develop following cure (Tripathi et al. 2008). Spleen provides an appropriate environment for the priming/activation of T and natural killer (NK) cells by antigen presenting cells (APCs) during L. donovani infection (Gorak et al. 1998). The infection stimulates rapid interleukin 12 (IL-12) p40 production by splenic dendritic cells (DCs) within the discrete T-cell zone in the periarteriolar lymphoid sheath (PALS) (Kaye, 1987). The appearance of IL-12 producing DCs in the PALS parallels an increase in high-affinity IL-2 receptors on CD4+ T cells, suggesting a classical model of DC-mediated T-cell activation (Gorak et al. 1998). In the spleen of L. donovani infected BALB/c mice, IL-10, IL-12, IFN-γ, and TGF-β are detected, although IL 4 remains absent (Melby et al. 2001). IFN-γ stimulates macrophages to produce iNOS catalysing formation of NO, a final effector molecule necessary for intracellular parasite killing. The animals treated with the combination of drug and vaccine showed the higher concentration of Th1-specific cytokines IFN-γ and IL-2 and lower concentration of Th2-specific cytokines IL-4 and IL-10 compared with SSG alone or 78 kDa alone with or without adjuvant, pointing towards the potential of immunochemotherapy to generate a protective immune response against L. donovani. Our results are in correspondence with the previous studies which showed that biological immunomodulators, such as interferon gamma (IFN-γ), enhance the activity of antimoniostatic cell-mediated immunity. Due to the existence of close association between chemotherapy and cell-mediated immunity, immunochemotherapy is thought to be more suitable for treatment of VL. Moreover, our study has shown that the use of specified antigens would result in a better approach to immunochemotherapy as they represent a higher and more specific type of immune response needed to cure the disease. However, more studies are required to test the combination in other animal models before it is tested in patients.
ACKNOWLEDGEMENTS

The authors acknowledge the support provided by the PURSE Grant of Department of Science and Technology, and University Grant Commission, Fellowship programme, India.

REFERENCES


