2. MATERIALS AND METHODS
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2.1. Collection of blood from kala-azar and post kala-azar dermal leishmaniasis patients

2.1.1. Collection of peripheral blood from patients

Forty KA patients admitted to the School of Tropical Medicine, Calcutta and the Canning Rural Hospital, West Bengal were included in this study. All these cases were diagnosed as KA after careful clinical and laboratory investigations and the diagnosis was confirmed by the demonstration of amastigotes (L. donovani bodies) in their bone marrow smears. The majority of these patients was found to be responsive to treatment with stibanate (sodium stibogluconate), but four patients did not respond to stibanate therapy and had to be treated subsequently with the drug pentamidine. Blood samples were collected from the KA patients before chemotherapy (with either stibanate or pentamidine). Convalescent-phase venous blood were collected from a few of these KA patients after successful chemotherapy. Blood samples were also collected from nine individuals who gave a history of having kala-azar 1-2 yr earlier. Venous blood were obtained from 16 patients suffering from post kala-azar dermal leishmaniasis as evidenced by the demonstration of amastigotes in their skin biopsy materials.

A limited number of blood samples were collected from healthy individuals living in areas where KA is known to be nonendemic and used as controls. Collected blood was allowed
to clot for overnight at 4°C and serum was separated by centrifugation (500 x g for 10 min). Sera, thus collected, were stored at -20°C until used.

2.1.2. Isolation of mononuclear cells from whole blood

Whole blood from KA patients was collected before the initiation of antileishmanial therapy. Blood samples were also obtained from healthy adults living in areas nonendemic for kala-azar. Heparinized venous blood of the study subjects was overlaid carefully on histopaque 1077 (Sigma, USA) taken in screw cap tubes, which were presiliconized using 'sigmacoat' (Sigma). Tubes were then centrifuged (300 x g) for 30 min at room temperature by following the methodology of Boyum (1968). Mononuclear cells, thus separated as a white band at the interface of histopaque layer (bottom) and plasma (top), were collected and resuspended in cold RPMI 1640 medium (Sigma)[supplemented with 0.2% (w/v) NaHCO3 (E. Merck, India), 2 mM L-glutamine (Sigma) and 50 μg/ml gentamicin (HI media, India)]. The cell suspension was then washed by centrifuging at 300 x g for 10 min at 4°C and pellet collected. The washing procedure was repeated twice more and finally, the pellet was resuspended in cold RPMI medium supplemented with 15% fetal bovine serum (FBS) (GIBCO, USA). Viability of the cells was determined by trypan blue dye exclusion method. For this, an aliquot portion (25 ul) of the cell suspension was appropriately diluted with an equal volume (25 ul) of 1% (w/v) of trypan blue (Sigma) solution in normal saline. The trypan blue containing cell suspension was
observed under a microscope (magnification, x 100). Viable cells (not stained with trypan blue) were counted by taking the sample in a hemocytometer (Ultraplane, Fuchs, USA). More than 95% of the cells in the suspension, thus counted, were found to be viable. Purity of the cell suspension was also checked by microscopic observation of the Giemsa (Sigma) (vide Appendix) stained smear of an aliquot portion of the mononuclear cell preparation.

2.2. Isolation and maintenance of *Leishmania donovani* parasites

2.2.1. Isolation of *L. donovani* strains

*Leishmania donovani* strain (BI2302) was isolated from the bone marrow culture of a kala-azar patient admitted to the Canning Rural Hospital, West Bengal. The bone marrow material was collected from the patient by the clinician (Dr. A. Nandy of School of Tropical Medicine, India) for confirmation of clinical diagnosis. Part of this material was smeared on a glass slide, stained with Giemsa and on examination under a microscope (magnification, x1000) showed the presence of amastigotes. Remaining part of the material was then inoculated intracardially into hamsters. Another part of the bone marrow material was inoculated to the "NNN" (Novy, MacNeal and Nicolle) medium (Nicolle, 1908b) (Table 2.1) for the isolation of parasites in the promastigote form grown *in vitro*. The *L. donovani* strain ASI, obtained from bone marrow material of a KA patient, was maintained *in vitro*
only through serial subcultures in "NNN" medium. Other L. donovani strains [Mohan, Sair (LD1), T1900 (LD2)] used in this study were collected from the bone marrow materials of KA patients. The L. donovani strain 2163 (LD3) was isolated from the skin biopsy material of a PKADL patient.

2.2.2. Maintenance of L. donovani parasites in vivo and in vitro

L. donovani strain (BI2302) was maintained in vivo through serial passages in Syrian (golden) hamsters (Mesocricetus auratus). These hamsters were kindly provided to us by Infar India Limited (Ganganagar, West Bengal, India). Thus, spleen(s) from heavily infected hamster(s) (6 to 8 weeks following infection via intracardial route) was homogenized by using a tissue homogenizer (Ten Broeck, Corning, USA) and the homogenate (containing amastigotes) was kept standing for a few minutes to allow the debris to settle. The supernatant, containing amastigotes, was used to infect a fresh batch of animals (hamsters) by intracardial inoculation. Whenever needed, a small portion of the hamster spleen material was collected under sterile condition and used to inoculate "NNN" medium. Promastigotes, thus obtained, were maintained in vitro through serial subcultures in "NNN" medium (if necessary). The strain of L. donovani (ASI) was maintained in vitro only through serial subcultures in "NNN" medium. The L. donovani strain (Mohan) was maintained in vivo through serial passages in hamsters. The other L. donovani strains (Sair, T1900, 2163) were maintained in vivo and/or
2.2.3. Preparation of "NNN" (Novy, MacNeal and Nicolle) medium

The "NNN" medium (Nicolle, 1908b) was prepared by mixing the ingredients given in Table 2.1.

Table 2.1 Composition of "NNN" medium for in vitro maintenance of L. donovani strains

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>0.6 gm</td>
</tr>
<tr>
<td>(Qualigens, India)</td>
<td></td>
</tr>
<tr>
<td>Agar powder</td>
<td>1.4 gm</td>
</tr>
<tr>
<td>(HI media)</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

The mixture was heated in a boiling water bath to dissolve the agar powder. Next, the molten medium was taken in a series of test tubes, each tube containing about 2 ml of the molten medium. The tubes were then cotton plugged and autoclaved at 15 lb pressure of steam for 15 min. Next, the tubes were allowed to cool to around 45°C and about 0.5 ml of defibrinated normal rabbit blood (sterilly collected) was added to each tube. After thorough mixing of the defibrinated blood with the agar-salt medium, the tubes were allowed to cool down further to make a solid slant. Gentamicin (HI media) was added to each tube to a final concentration of 50
ug/ml. The tubes (containing "NNN" medium) were then kept at 4°C until used.

2.3. Establishment of *Leishmania donovani* infection in BALB/c mice

2.3.1. Infection of BALB/c mice with *L. donovani* amastigotes

(a) Source of animals

Laboratory inbred BALB/c strain of mice of 20-25 gm body weight (6-8 weeks, male), [purchased from the National Institute of Nutrition, Hyderabad, India], were used in all experiments. All animals received boiled water *ad libitum* and nutritionally balanced food pellet diet (Lipton India Limited, India).

(b) Inoculation of BALB/c mice with leishmanial amastigotes

Initially, a few hamsters were inoculated via intracardial route with the bone marrow material (containing amastigotes) derived from a kala-azar patient (vide Section 2.2.1). About eight weeks later, one hamster from this group was sacrificed, its spleen removed surgically, teased gently over a fine metal screen (80 mesh, Sigma) and finally, homogenised by using a tissue homogeniser (Ten Broeck, Corning). The splenic homogenate, taken in cold normal (0.85%) saline, was centrifuged at low speed (70 x g) for 2 min to remove debris. The supernatant was collected and subjected to centrifugation at 1500 x g for 10 min. The pellet, containing amastigotes, was resuspended in cold normal saline to yield a suspension containing about 1-2 x
$10^8$ parasites/ml (strain BI2302). This parasite suspension was used to infect a fresh batch of BALB/c (about 25 to 30 in number) mice by the intracardial route of inoculation (0.1 ml of suspension/mouse).

2.3.2. Determination of parasite load in the spleen and liver of infected mice

Amastigotes were detected and counted in the impression smears of spleen and liver of infected mice (following sacrifice) by the method of Stauber et al (1958). Several impression smears were taken by gently pressing the cut surface of liver and/or spleen tissue sections on clean (grease free) microscopic glass slides. Slides, with the impression smears, were air dried, fixed with absolute methanol (Qualigens) and stained with Giemsa (Sigma). Stained smears were then washed with 0.2 M phosphate buffer (pH 7.4) (vide Appendix) and the slides were air dried. Stained slides were examined under a microscope (Leitz, Model No. 470462, Germany) using a magnification, x 1000.

Parasite load in the organs (liver and spleen) of the infected animals was determined by counting the number of amastigotes per, at least, 500 nucleated cells in the impression smear. Total parasite load in the organ was determined using the following equation used by Stauber (1958).
Total parasite load (in organ) =

\[
\frac{\text{Number of amastigotes}}{\text{Number of nucleated cells}} \times \text{Organ weight (mg)} \times 2 \times 10^5
\]

Organ (liver and spleen) weights of normal and \textit{L. donovani} infected BALB/c mouse (collected surgically after their sacrifice) was determined by using an analytical single pan balance (K. Roy analytical single pan balance, K-15 Super, India).

2.3.3. Collection of sera from infected mice

Blood samples were collected by cardiac puncture from normal and infected mice after sacrifice. About 0.4 ml of blood was allowed to clot for overnight at 4°C. Serum was separated by centrifugation (500 \times g) for 10 min, collected and stored at -20°C until used.

2.4. Histopathologic examination of liver tissue sections of normal and \textit{L. donovani} infected mice

2.4.1. Preparation of tissue sections

Tissue (liver) fragments, collected from normal and infected BALB/c mice following their sacrifice, were immediately fixed in Bouin’s fluid (Sheehan and Hrapchak, 1980) (vide Appendix). Fixed tissue fragments were gradually dehydrated by successive treatment with 30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v) and 99.99% (v/v) (absolute) alcoholic solution (in distilled water) and finally, with xylene. The dehydrated tissue fragments were preserved under cedarwood oil (Flatters and Garnett Limited, England) at room
temperature. Whenever needed, preserved tissue fragments were treated with xylene for 2 to 3 min to remove the residual cedarwood oil and then immersed in a 1:1 mixture of molten paraffin (m.p. 58°C to 60°C; E. Merck, India). After about 30 min, tissue fragments were carefully taken out from molten paraffin-xylene mixture, transferred to another mixture of paraffin (75%) and xylene (25%) and incubated at 65°C for 2 h. Finally, tissue material was embedded carefully on molten paraffin and allowed to solidify. Tissue blocks, thus obtained, were taken for sectioning. For this, each block was trimmed, fixed on a metal block holder and the holder was attached to a rotary microtome (Optex brand, Japan). Tissue sections (of about 5 um thickness) were then cut serially using the rotary microtome. Next, serial tissue sections in wax ribbon were stretched by carefully placing the ribbon (containing tissue sections) in lukewarm water. Stretched tissue sections were then placed over a microscopic glass slide containing a thin coating of Mayer’s egg albumin (vide Appendix) as fixative. Slides were then kept at 37°C in an incubator for 3 to 4 days following which tissue sections became ready for staining.

2.4.2 Staining of the tissue sections with haematoxylin and eosin

The slides containing several tissue sections were treated first with xylene for 2-3 min and then, with a graded series of alcoholic solution [99.99% (v/v) to 30% (v/v)] in water. Slides were kept for, at least, 5 min in each grade of
alcoholic solution. Following treatment with 30% (v/v) alcoholic solution, slides (containing tissue sections) were immersed in water for a few min and stained with hematoxylin (Qualigen). Stained slides were then washed with tap water and subsequently, processed in a series of graded alcoholic solution [30% (v/v) to 70% (v/v)] in water. Finally, slides were stained with a 2% (w/v) eosin (S.D. Fine Chemicals, India) solution in 70% (v/v) alcohol. Eosin stained slides were washed initially, with 90% alcohol and then, with absolute alcohol. Next, stained slides were treated with fresh xylene and then with a mounting agent (Entellan; E. Merck). Cover slips (Blue star, India) were then carefully laid over the stained tissue sections (covered with the mounting agent) on the slide and allowed to dry. Permanent slides of tissue sections, thus prepared, were observed under a microscope (Leitz, Germany) using appropriate magnifications.

2.5 Treatment of *L. donovani* infected BALB/c mice with the drug sodium stibogluconate (stibanate)

A group of BALB/c mice (about 12 in number), infected with *L. donovani* amastigotes, were treated with the antileishmanial drug sodium stibogluconate (stibanate) (100 mg Sb\textsuperscript{V}/kg body weight/dose). Each mouse received five successive intramuscular injections (course I) on every alternate day starting from day 15 following infection. One month following completion of the 1st course (each course consisted of five such injections) of stibanate therapy, three mice from this group were sacrificed and the parasite load in their spleen/liver was determined as
described earlier (vide Section 2.3.2). The immune status of these mice was studied by serology (described in Section 2.9) as well as lymphocyte transformation experiments (vide Section 2.11). The remaining mice were given another course (course II) of stibanate treatment following an interval of 15 days from the date of last stibanate injection in course I. The parasite load in the liver/spleen of these treated mice was monitored 2, 4 and 6 months after the chemotherapy i.e. completion of course II of stibanate treatment.

2.6 Large scale cultivation of *L. donovani* promastigotes

2.6.1 Cultivation of *L. donovani* promastigotes in an enriched solid medium

*L. donovani* promastigotes (strain ASI) were cultivated in Ray’s medium (Ray, 1932) using appropriate modifications (Nandy, *et al.* 1987). The composition of the modified Ray’s medium is given in Table 2.2.

Table 2.2 Composition of the modified Ray’s medium for the cultivation of *L. donovani* promastigotes

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain heart infusion (Difco, USA)</td>
<td>2.8 gm</td>
</tr>
<tr>
<td>Bactopeptone (Difco)</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>D-Glucose (Qualigens)</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Agar powder (Hi media)</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Initially, the first three ingredients (except the agar powder) were dissolved in distilled water and pH of the mixture was adjusted to 7.2 by 1 N sodium hydroxide (E. Merck) solution. Next, agar powder was added and the mixture was then heated on a boiling water bath to dissolve the agar. The agar medium, thus prepared, was distributed to a series of test tubes, each tube containing about 9 ml of the medium. Tubes, containing the medium, were then cotton plugged and autoclaved under steam at 10 lb pressure for 10 min. Next, the tubes (with the medium) were allowed to cool down and about 1.0 ml of defibrinated normal rabbit blood was added to each tube (tubes being maintained at a temperature of about 45°C to 48°C) and the contents mixed well. Tubes were then kept in an inclined position to form a slant on cooling. Next, gentamicin (HI media) was added to each tube to a final concentration of 50 μg/ml. Medium containing tubes were then kept at 4°C until used.

Culture tubes were inoculated with *Leishmania donovani* promastigotes by streaking a loopful inoculum on the surface of the solid medium using an inoculation loop needle. Inoculated culture tubes were incubated for about 7 days at 23 ± 0.5°C in a B.O.D. incubator (Amalgamated Suppliers, India). *L. donovani* promastigotes, grown as a thick mucous layer on the surface of the solid medium, were harvested by gentle washing with normal saline.
2.6.2 Cultivation of *L. donovani* promastigotes in a liquid medium

*L. donovani* promastigotes were grown in F-12 (Sigma) liquid medium containing certain other ingredients as developed in this study (Table 2.3). Rabbit blood cell lysate, used as one of the ingredients for this liquid medium, was prepared as follows:

(a) Preparation of rabbit blood cell lysate

Normal rabbit blood was collected by heart puncture. Defibrinated blood was diluted with distilled water (1:10) and allowed to stand at 4 °C for 30 min. Lysed blood was then centrifuged at 10,000 x g for 20 min to remove cell stroma and supernatant collected. Finally, the lysate was made 0.15 M with respect to sodium chloride by the addition of a calculated amount of salt and stored frozen at -20 °C until used.

The liquid culture medium was prepared by mixing the ingredients in the proportions cited in Table 2.3.

(b) Table 2.3 Composition* of the liquid culture medium for the cultivation of *L. donovani* promastigotes

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amounts (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-12 (10X)</td>
<td>10</td>
</tr>
<tr>
<td>(Sigma)</td>
<td></td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>5</td>
</tr>
<tr>
<td>(Inovar, USA)</td>
<td></td>
</tr>
<tr>
<td>Normal rabbit blood lysate</td>
<td>10</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>75</td>
</tr>
</tbody>
</table>
* Following mixing of the added ingredients, sodium bicarbonate was added (1.2 gm/lit of medium) to the liquid medium and pH was adjusted to 7.2 to 7.4 by the addition of 1 N sodium hydroxide. Gentamicin (HI media) was added to the medium to a final concentration of 50 μg/ml. Finally, the medium was sterilised by filtration using 0.45 μm pore size filter disc (Millipore Corp., USA).

(c) Cultural conditions for promastigotes

About 10 ml of the medium was dispensed in each of a series of sterile cotton plugged flasks (100 ml). The flasks were inoculated with *L. donovani* promastigotes (about 1-2 x 10⁶ cells/ml of culture). Inoculated flasks were incubated for a period of 5-6 days in a B.O.D. incubator at 23 ± 0.5 °C. Growth, purity and viability of the parasites in the culture medium was checked by taking a drop of the culture on a clean microscopic glass slide and observed under a microscope (Olympus, Japan) using a magnification of x 400. Another aliquot portion of the culture medium (containing parasites) was appropriately diluted in 1.0% (v/v) formalin solution in normal saline. A portion of this diluted parasite suspension was taken on a hemocytometer (Ultraplane, Fuchs, USA) and the parasite number was determined by counting under a microscope (Olympus) using a magnification of x 400. A loopfull amount of the parasite culture was smeared on a glass slide and stained with Giemsa to check the purity of promastigote culture (Fig. 2.1).
Fig. 2.1 *Leishmania donovani* promastigotes stained with Giemsa (x 2720).
2.7. Preparation of antigens from *L. donovani* promastigotes

2.7.1 Preparation of heat-killed (HK) promastigote antigen

*Leishmania donovani* promastigotes were grown in the enriched liquid culture medium (containing F-12 as one of the ingredients) (vide Section 2.6.2). Promastigotes, during their logarithmic phase of growth (4-5 days), were harvested by centrifugation (500 x g) for 15 min at 4°C. The parasite pellet was washed by resuspending in normal saline (0.85%) and centrifuging (500 x g) for 15 min at 4°C. The washing procedure was repeated twice. Finally, the washed parasites were resuspended in normal saline so that the suspension contained about 2 x 10^8 promastigotes/ml). Next, the parasite suspension was heated to 56°C for 1 hr in a water-bath (Howard, et al, 1984). Viability of the promastigote suspension, following heat treatment, was checked by inoculating a small portion of the parasite suspension to "NNN" medium and/or liquid medium. Another small portion, taken on a glass slide, was observed under a microscope (magnification, x 400). No viable parasites were demonstrable by either of these procedures. The morphology of the heat killed promastigotes was, however, found to be altered to round shaped bodies with some degenerated parasites being demonstrable.

2.7.2 Preparation of freeze-thawed antigen

Promastigote suspension (2 x 10^8 cells/ml of normal saline) was subjected to 5 cycles of freezing and thawing by
keeping the material alternatively at -40 °C for 2 h and 37 °C for another 2 h respectively. Viability of the freeze-thawed promastigotes, thus prepared, was examined as described earlier (vide Section 2.7.1). No viable parasites were demonstrable following microscopic examination. The material was then kept at -20 °C until used.

2.7.3 Preparation of formalin-killed (FK) promastigote antigen

_Leishmania donovani_ promastigotes were grown and harvested by the same procedure as described in Section 2.7.1. Promastigotes were resuspended (2 x 10⁸ parasites/ml of suspension) in 1.0 ml of normal saline and treated with formalin (Qualigen, India) so that the final concentration of formalin was 0.1% (v/v) in the suspension mixture (Holbrook _et al_, 1981). The promastigote suspension in 0.1% (v/v) formalin saline solution was then kept at 4 °C for overnight. Next, formalin treated promastigotes were harvested by centrifugation at (500 x g) for 15 min and washed twice with cold normal saline. The final pellet was resuspended in normal saline and the number of promastigote in the suspension was adjusted to 2 x 10⁸ cells/ml. Viability of the formalin treated parasites was checked by the procedure as described earlier (vide Section 2.7.1). No viable cells were demonstrable. Morphology of the formalin-killed promastigotes remained, essentially, unaltered when examined under a microscope (magnification, x 400).
2.7.4 Preparation of sonicated-killed (SK) promastigote antigen

Promastigotes were grown and harvested by the same procedure as described in Section 2.7.1. The promastigote suspension (~ $2 \times 10^8$ cells/ml) in saline was sonicated at 4°C by an ultrasonicator (Braunsonic 1510, B. Braun AG, Germany). Six to seven pulses, each of 30 sec duration (using an output power of 100 watt), were used for disruption of the parasites. Viability of the parasites was checked as described earlier (vide Section 2.7.1). No viable parasites were demonstrable. However, the morphology of the parasites was totally lost and parasites became degenerated. Only a few dead promastigote bodies were detected when the sonicated material was observed under a microscope (magnification, x 400).

2.7.5 Preparation of crude soluble antigen (CSA) from promastigotes

The crude soluble antigen (CSA) was prepared from *L. donovani* promastigotes (strain ASI) maintained and cultivated in modified Ray’s medium (vide Section 2.6.1). Promastigotes, grown as thick mucous layer on the surface of the solid medium, were harvested by gentle washing with normal (0.85%) saline. Harvested parasite suspension was centrifuged at 500 x g for 15 min and the supernatant was discarded. The parasite pellet was resuspended again in normal saline and centrifuged. The washing procedure was repeated twice. Finally, the parasite pellet was resuspended in 10 mM Tris-
HCl buffer (pH 7.4) containing 20 mM sodium chloride, 10 mM ethylenediamine tetraacetic acid (EDTA) disodium salt (Glaxo, India), 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 2 mM iodoacetamide (Sigma) and 50uM leupeptin (Sigma). Number of promastigotes in the resuspended material was adjusted to about 1 x 10^9 cells/ml. Leishmanial crude soluble antigen was prepared, essentially, by the method of Ghose et al (1980). For this, about 5 ml of the parasite suspension was homogenized with a Dounce homogeniser (Wheaton, USA) in cold (4 °C) using 15 to 20 strokes. Next, the homogenized material was further sonicated at 4 °C by using an ultrasonicator (Braunsonic 1510, B. Braun AG, Germany) using 6 to 7 pulses each of 30 sec duration (an output power of 100 watt was used for sonication). The sonicated material was centrifuged at 6,000 x g for 15 min at 4 °C and the clear supernatant was collected as the CSA.

2.8. Analytical methods

2.8.1. Protein estimation

Protein content of the test sample was estimated by the modified Lowry’s method (Markwell et al, 1978). For this, protein regents A and B (vide Appendix) were mixed in the ratio of 100:1 and 3 ml of the mixture was added to 1 ml of the appropriately diluted test sample. The mixture was incubated for 30 min at room temperature. Finally, colour was developed by the addition of 0.3 ml of Folin and Ciocalteu’s reagent (SRL, India) to each tube containing the sample mixture and subsequent incubation at room temperature for
another 30 min. Intensity of colour developed was measured by determination of the optical density (OD) of the mixture at 660 nm by a spectrophotometer (Shimadzu, Model No. UV-240, Japan). Protein content of the test sample was determined from the OD value derived as above and plotting this value on a standard curve drawn with known amounts of bovine serum albumin (BSA) (Sigma) (Fig. 2.2).

2.8.2. Analysis of leishmanial antigens by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Test samples were electrophoresed in SDS-polyacrylamide gel by the method of Laemmli (1970).

(a) Preparation of the gel slab. Gel was cast between glass plates (15 x 15 cm) to a height of 10 cm using a spacer of 1 mm thickness. About 18 ml of the gel mixture [containing 12.5% (w/v) acrylamide (Sigma), 0.33% (w/v) N,N'-methylene-bis-acrylamide (Sigma), 0.1% (w/v) sodium dodecyl sulphate (SDS) (Sigma), 0.38% (w/v) potassium persulphate (Sigma), 0.06% (v/v) of N,N,N',N'-tetramethyl ethylene diamine (TEMED) (Sigma) in 1.5 M Tris-HCl buffer, pH 8.8] was used to prepare the gel which was overlaid with a few drops of water. After polymerization, the water layer at the top of the gel was removed and stacking gel mixture [containing 4.5% (w/v) of acrylamide, 0.12% (w/v) of N,N'-methylene-bis-acrylamide, 0.1% (w/v) of SDS, 0.03% (w/v) of potassium persulphate, 0.1% (v/v) TEMED in 0.5 M Tris-HCl buffer, pH 6.8] was applied on the top of the separating gel. A comb was then inserted into the stacking gel mixture and the gel was allowed to
Fig. 2.2. Standard curve with bovine serum albumin for the estimation of protein content of test samples.
polymerise. Following polymerization of the stacking gel, the comb was removed and slots, thus formed, were washed with electrode buffer, pH 8.3.

(b) Electrode buffer. The electrode buffer contained 0.025 M of Tris (SRL, India), 0.192 M of glycine (SRL, India) and 0.1% (w/v) of SDS (Sigma). Final pH of the buffer, if necessary, was adjusted to 8.3 with 1 N HCl.

Preparation of test sample for electrophoresis. Test sample for SDS-PAGE was prepared from freshly harvested _L. donovani_ promastigotes. For this, promastigotes were grown for 4 to 5 days in a liquid medium (containing F-12) (vide Section 2.6.2). Harvested and washed promastigotes (about 3.5 x 10^7 cells) were resuspended in 200 ul of distilled water. Next, 200 μl of sample buffer [10 mM of Tris-HCl, pH 6.8, containing 2% SDS (w/v) (Sigma), 5% (v/v) 2-mercaptoethanol (Sigma), 10% (w/v) sucrose (SRL, India), 1 mM of EDTA-disodium salt (Qualigens) and 0.002% (w/v) bromophenol blue (Qualigen)] was added to the parasite suspension. The suspension was then heated in a boiling water bath for about 10-15 min and allowed to cool down to room temperature. The sample (solubilized promastigotes) for electrophoresis, thus prepared, was applied to the lanes of a 12.5% polyacrylamide gel as prepared earlier in this section (vide Section 2.8.2a). For certain experiments, CSA of _L. donovani_ promastigotes was also subjected to SDS-PAGE analysis.
(c) Electrophoresis. Test samples (solubilized *L. donovani* promastigote antigen or CSA), prepared as above, were electrophoresed in a vertical slab gel unit (Atto Corp., Japan) at room temperature by applying a constant voltage (60 volts initially for about 15 to 20 min which was subsequently increased to 120 volts). Electrophoresis continued, usually, for 4.5 h till the tracking dye reached approximately 1 cm above the bottom of the gel. For each electrophoresis run, one lane contained a mixture of marker proteins (Sigma) of known subunit molecular weights such as, bovine serum albumin (66,000 daltons), ovalbumin (45,000 daltons), glyceraldehyde 3-phosphate dehydrogenase (36,000 daltons), carbonic anhydrase (29,000 daltons), trypsinogen (24,000 daltons) and α-lactalbumin (14,200 daltons).

(d) Staining of gels

Coomassie staining. Following electrophoresis, gels were carefully transferred from the glass plate to a fixative solution containing 50% (v/v) methanol and 10% (v/v) acetic acid in water and kept for 16-18 h at room temperature. After removal of the fixative solution, gels were stained with 0.25% (w/v) solution of Coommasie Brilliant blue R250 (SRL, India) dissolved in the same fixative solution. Finally, gels were destained by washing with a solution of 5% (v/v) methanol and 7.5% (v/v) acetic acid in water until the background became colourless.

Silver staining. Following SDS-PAGE, gels were silver stained for proteins by the method of Blum et al. (1987). For
this, gels were fixed in 50% (v/v) methanol and 12% (v/v) acetic acid in deionised water for overnight (about 16 hr). After removal of the fixative solution, gels were washed with 50% ethanol for 10 min. The washing procedure was repeated thrice. Following washing, gels were treated with a solution of 0.2% (w/v) sodium thiosulphate (Qualigens) in deionized water for 1 min. Next, gels were washed with deionised water and treated with 0.02% (w/v) silver nitrate (SRL, India) solution containing 0.075% (v/v) formalin for 20 min with shaking. After washing with deionised water, gels were immersed in the developer solution containing 6% (w/v) sodium carbonate (Qualigens), 0.05 (v/v) formalin and a few crystals of sodium thiosulphate with constant shaking till the appearance of the silver stained bands. The gels were incubated at room temperature until the stained bands reached the desired intensity. To stop the reaction, a fixative solution containing 50% (v/v) methanol and 12% (v/v) acetic acid was added to the gel. Stained gels were stored in 5% (v/v) acetic acid solution in water.

(e) Determination of subunit molecular weight of proteins.

For the determination of subunit molecular weight of proteins, a standard curve (Fig 2.3) was constructed by plotting the relative electrophoretic mobilities (Rf) of the marker proteins against the logarithm of their corresponding subunit molecular weights. The Rf value was determined according to Weber and Osborn (1969) by using the following formula.
Fig. 2.3. Relative mobilities of standard marker proteins in slab-gel electrophoresis.
Distance migrated by the protein (after staining and destaining)  
\[
\text{Rf} = \frac{\text{Distance migrated by the tracking dye before staining}}{\text{Length of the gel after staining and destaining}} \times \frac{\text{Length of the gel before staining}}{\text{Length of the gel after staining and destaining}}
\]

The subunit molecular weight of proteins present in the test samples was determined by comparing their Rf values with those of marker proteins used to construct the standard curve.

2.9. Serological techniques

2.9.1 Enzyme-linked immunosorbent assay (ELISA)

The micro-ELISA method was used for the determination of antileishmanial antibody titers in KA and PKADL sera (Engvall and Perlman, 1971). For this, wells of micro-ELISA Maxisorp plates (NUNC, Denmark) were sensitized by overnight incubation at 4°C with 5 \( \mu \text{g} \) (each well) of CSA protein in 100 \( \mu \text{l} \) of carbonate-bicarbonate buffer (vide Appendix). Following overnight incubation at 4°C, the plate was washed three times with 0.15 M phosphate-buffered saline (PBS), pH 7.4, containing 0.05% (v/v) Tween 20 (Sigma) (PBS-Tween) (vide Appendix). Next, uncoated sites of the wells were blocked by the addition of 200 \( \mu \text{l} \) of a 3% (w/v) solution of BSA (Sigma) in PBS. Following incubation for 2 h at room temperature, the wells of the plate were washed four times with PBS-Tween solution. Next, 100 \( \mu \text{l} \) aliquot of test serum, serially twofold diluted with a solution of 0.5% (w/v) BSA in PBS, was added to each well and incubated for 2 h at room temperature.
Next, the wells were repeatedly (at least 4 to 5 times) washed with PBS-Tween solution, following which 100 μl of goat antihuman IgG or IgE peroxidase conjugate (Sigma), appropriately diluted in 0.05% (w/v) BSA-PBS, was added to each well. Next, the plate was incubated for 1 h at room temperature followed by successive washing with PBS-Tween solution. Finally, 100 μl of the substrate solution [o-phenylenediamine (Sigma) (1 mg/ml)] in 0.1 M citrate buffer (pH 4.5) (vide Appendix) and 30% (v/v) hydrogen peroxide (1 μl/2 ml)] was added to each well and the plate was incubated for about 10 min at room temperature. Next, the reaction was stopped by the addition of a 5 N sulphuric acid solution (100 μl/well) and the optical density of the colour developed in each well of the microtitre plate was determined at 492 nm by using a micro-ELISA reader (Anthos 2001, Austria). A background control (substrate only) and a negative control (containing serially diluted normal human serum) were included in each set of experiments. The ELISA titre was expressed as the reciprocal of highest dilution of antiserum that showed definite colour development (an A₄₉₂ of ≥ 0.2) in the assay.

In certain experiments, whole promastigotes were also used as the coating antigen in IgG-ELISA. For this, freshly harvested promastigotes, grown in the enriched F-12 liquid medium (vide Section 2.6.2) were resuspended in the coating buffer at a concentration of ~1 x 10⁷ per ml and 100 μl of this cell suspension was added to each well of the micro-
ELISA plates. The rest of the assay procedure was the same as described in the above paragraphs.

IgG subclass-specific antileishmanial antibody responses in the sera of active and treated KA and PKADL patients were also measured in microtiteration plates with CSA as the antigen. The procedure followed was very similar to that described above except that instead of the peroxidase-antibody conjugate, 100 µl of appropriately diluted biotin-conjugated mouse monoclonal antibodies to human IgG1/IgG2/IgG3 or IgG4 (Sigma) were added to each well for isotype-specific antibody measurements. Following incubation and washing with PBS-Tween, 100 µl of streptavidin-peroxidase (Sigma), appropriately diluted with PBS-0.05% BSA, was added. This was followed by the addition of the substrate solution. Results were recorded as described above.

Antileishmanial antibody titres in the sera of infected or immunized BALB/c mice were also determined by the micro-ELISA experiments. The procedure was identical to that described above except that antimouse IgG peroxidase conjugate (Sigma) was used instead of the antihuman conjugates.

2.9.2 Immunoblotting

The reactivity of the IgG class and subclasses of antileishmanial antibodies in active and treated KA and PKADL sera to promastigote antigens was studied by immunoblot experiments. The methodology of Towbin et al (1979) was
followed with minor modifications (Sengupta et al, 1989). The immunoblot technique adopted for the purpose can be divided into three major steps: (a) SDS-PAGE of the test sample, (b) electrophoretic transfer and (c) immunostaining.

(a) SDS-PAGE. For this, *L. donovani* whole promastigote antigen (~1 x 10⁷ parasites per lane) or CSA (100 µg of protein/lane) was separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970) as described in the Section 2.8.2 a-c.

(b) Electrophoretic transfer. Following electrophoresis, the gel was removed from the casting plates and equilibrated with the blotting buffer [containing 25 mM of Tris-HCl, 192 mM of glycine (SRL, India) and 20% (v/v) methanol (E. Merck); pH 8.3] for about 10 min. Following equilibration, the electrophoresed gel was transferred to thick scotch-brite pads which were presoaked with blotting buffer for 20 min. Next, nitrocellulose paper (pore size 0.2 µm, Sigma) of appropriate size moistened in the same buffer were carefully placed on the surface of the gel (containing the electrophoresed material) to avoid any trapped air bubbles. Several layers of the presoaked pads were then placed over the membranes. The complete sandwich (keeping the membranes facing cathode) was then put into a transblot apparatus (Atto Corp., Japan) and the latter was subsequently filled in with the blotting buffer. Antigen components separated in the electrophoresed gel were then transferred electrophoretically.
onto the nitrocellulose strips at room temperature by applying a constant current of 170 mA for about two hours. The blotted strip containing marker proteins was stained with amido black [0.1% (w/v) of amido black in an aqueous solution of 10% (v/v) glacial acetic acid]. Stained strip was then destained with a destainer solution [2% (v/v) of glacial acetic acid in distilled water].

(c) Immunostaining. For immunostaining, the blotted strips containing separated antigens were washed with 10 mM Tris-HCl buffer (pH 7.4) and treated with a 3% (w/v) BSA solution in PBS for overnight. After washing with PBS-Tween, the strips were incubated with appropriate dilutions of test serum for 2 h at room temperature. Following incubation, strips were washed with the same PBS-Tween solution and subsequently treated with either goat antihuman IgG peroxidase conjugate (Sigma) (diluted with 0.5% (w/v) of BSA solution in PBS). Following incubation for 1 h at room temperature, strips were washed and finally, developed by the treatment with the substrate solution of 3,3'-diaminobenzidine hydrochloride (DAB)(Sigma) [1 mg/ml of DAB in Tris-HCl buffer (pH 7.4) containing 200 mM sodium chloride and 30% (v/v) solution of $H_2O_2$].

Immunoblot experiments using the sera of *L. donovani* infected BALB/c mice was also carried out. The procedure was identical to that described above except that antimouse IgG peroxidase conjugate was used instead of the antihuman conjugate.
Immunoblot analysis of leishmanial antigens was also carried out by using patients’ sera and reagents specific for different subclasses of human IgG. The methodology was essentially similar to that described above except that following the incubation with patients’ sera, the strips were washed and treated with biotinylated mouse monoclonal antibodies to human IgG1, IgG2, IgG3 and IgG4 (Sigma) and incubated for 1 h at room temperature. Next, the strips were washed and treated with a streptavidine-peroxidase conjugate (Sigma) (diluted to 1:10,000 with 0.5% BSA solution in PBS). Following incubation for another 1 h, the strips were washed and finally, developed by the treatment with the substrate solution as described above.

2.10 Quantitation of subpopulations of T lymphocytes in the peripheral blood of KA patients

Blood from KA patients were collected before initiation of chemotherapy as well as following completion of treatment. Blood samples were also collected from healthy individuals and used as controls. Peripheral blood mononuclear cells were isolated by density gradient centrifugation as described in Section 2.1.2. Cells were washed and finally, resuspended (5 x 10^6 cells/ml) in HAM’s F-12 cell culture medium (Sigma) supplemented with 10% FBS. Surface phenotypes of the lymphocytes were determined by indirect immunofluorescence technique using mouse monoclonal antibodies to CD2, CD4 and CD8 antigens (Dakopatts A/S, Denmark) following the manufacturer’s instructions. To about 5 x 10^5 cells in 100 μl
suspensions, primary antibodies were added. After 30 min of
incubation and washing, fluorescein isothiocyanate (FITC)-
conjugated rabbit antimouse antibodies were added. Following
fluorescein labelling of cells separately for each phenotype,
smears of cell suspension were made on glass slides, fixed
with methanol and mounted in PBS (pH 7.4) containing 30%
glycerol. Percentage of stained cells developed with each
marker reagent was determined by examining and counting of
the total number of cells in a given field, first under
fluorescence followed by phase contrast microscopy (Zeiss
Axioscope Fluorescence Microscope, Germany), using
appropriate filters for FITC conjugates. For each
determination, a total of, at least, 200 cells was counted
and the percentage of cells bearing each phenotype was
calculated. The total number of cells bearing a given
phenotype in the peripheral blood of the subjects was
determined by using these values as well as total and
differential white blood cell counts made earlier.

2.11. Determination of lymphoproliferative response in vitro
(a) Preparation of mononuclear cells

Peripheral blood mononuclear cells were prepared from
heparinised venous blood collected from active KA patients as
well as normal individuals as described in the Section 2.1.2.

Mononuclear cells from BALB/c mouse spleen were also
isolated for lymphocyte transformation experiments. For this,
animals (normal, infected or immunized) were sacrificed and
their spleen removed surgically, homogenized separately in cold RPMI 1640 medium by using a tissue homogenizer (Ten Broeck, Corning). Homogenized cell suspension was overlaid on histopaque 1077 and processed as described above. Lymphocyte enriched mononuclear cells, thus obtained, were counted and checked for their viability as described above (vide Section 2.1.2).

(b) Mitogen induced lymphocyte transformation experiments in vitro

Mononuclear cells obtained either from the peripheral blood of KA patients or from the spleen of sacrificed BALB/c mice (total spleen mononuclear cell suspension) were adjusted to a final concentration of ~2 x 10^6 cells/ml of the suspension by RPMI 1640 medium supplemented with 15% FBS (Gibco, USA). The cell suspension was then distributed into a 96 well tissue culture plate (Corning) so that, each well contained about 2 x 10^5 cells/100 μl of cell suspension. Next, cells present in the wells were stimulated by the addition of a solution of mitogen phytohaemagglutinin P (PHA) (Sigma) and/or concanavalin A (Con A)(Sigma) so that the final concentrations of the mitogen in the cell suspension was 0.5 μg/ml and 2.5 μg/ml. Final volume of the culture fluid in each of the wells was adjusted to 200 μl with RPMI 1640 (containing 15% FBS) medium. Tissue culture plates containing lymphocytes were incubated for 48 h at 37°C in air containing 5% carbon dioxide (CO₂) in a CO₂ incubator (Nuaire, USA). Next, [³H]-thymidine (Amersham, UK), diluted
with RPMI 1640 medium, was added to each well (0.5 μCi/25 μl/well) of the plate and cells were incubated for a further period of 20 h. Finally, cells were harvested on glass fibre filters (Whatman, UK) using a cell harvester (NUNC, Denmark). Uptake of the [³H]-thymidine by cells was measured in a Liquid Scintillation Counter (Wallac, Pharmacia, Sweden).

Each experimental determination was run in triplicate with appropriate control (unstimulated) cell cultures. Lymphocyte transformation index (LTI) was determined by using the relation:

\[
\frac{\text{count per min (cpm) of stimulated cultures}}{\text{count per min (cpm) of unstimulated cultures}}
\]

and [³H-thymidine incorporation (cpm) was expressed as:

\[ \Delta \text{cpm} = (\text{count per min of stimulated cultures}) - (\text{count per min of unstimulated cultures}) \]

(c) Leishmanial antigen induced lymphocyte transformation experiments in vitro

Lymphocytes isolated from peripheral blood of human subjects or spleen of BALB/c mice were also stimulated with different concentrations of leishmanial freeze-thawed antigen (vide Section 2.7.2). For this, cells present in each well of the tissue culture plate were stimulated with 1 x 10⁶ equivalent promastigote material and/or 5 x 10⁶ equivalent promastigote material/ml. Following 96 h incubation in the CO₂ incubator, [³H]-thymidine was added to each well (0.5 μCi/well) and the cultures were further incubated for another 20 h. Next, cells were harvested and counted in a similar way
as described earlier in this section.

(d) Fractionated promastigote antigen induced lymphocyte transformation experiments in vitro (T cell immunoblotting)

Preparation of fractionated antigen. Antigens recognized by T lymphocytes were examined by the technique of T cell immunoblotting using the methodology of Abou-Zeid et al (1987). The technique described below is separated into three major steps:

SDS-PAGE of the test sample. About $1 \times 10^9 \text{ L. donovani}$ promastigotes (ASI) and/or 2 mg protein of its CSA preparation were electrophoresed in SDS-polyacrylamide gel as described in the Section 2.8.2a.

Electroblotting. Following electrophoresis, separated antigens were transblotted onto nitrocellulose membrane (vide Section 2.9.2.b) following the methodology of Towbin et al (1979).

Excision and preparation of fractionated antigens. After electrophoretic transfer of separated antigens onto nitrocellulose membrane, a vertical section of the blotted membrane was stained with 0.1% amido black to identify protein bands of interest. The rest part of the blotted membrane (containing transferred leishmanial antigen fractions) was appropriately cut into horizontal sections to separate the desired antigen bands. Further, the horizontal sections containing desired antigen fractions were minced separately and dissolved in dimethyl sulfoxide (DMSO) (Sigma)
for 30 min at room temperature. The antigen-bearing particles were precipitated by the gradual addition of 50 mM carbonate-bicarbonate buffer, pH 9.6 (vide Appendix). Next, the particulate antigens were pelleted by centrifugation at 10,000 x g for 20 min at 4°C. The pellet was resuspended in normal saline and washed extensively by centrifugation (10,000 x g for 20 min) at 4°C. Following washing with normal saline, particulate antigens were resuspended in 2 ml RPMI-1640 medium (Sigma, USA), pH 7.2-7.4 containing 2 gm/lit sodium bicarbonate (E. Merck, India), 2 mM L-glutamine (Sigma) and a cocktail of 100 μg/ml streptomycin (Sigma), 100 U/ml penicillin (Sigma) and 50 μg/ml gentamicin (Hi media) and stored at -20°C until used. Graded doses of this particulate antigenic preparations were used in lymphoproliferative assays.

Lymphocyte transformation experiments in vitro. Splenic lymphocytes, obtained from infected and drug treated BALB/c mice, were distributed in 96 well tissue culture plates as described above. Lymphocytes in the tissue culture wells were stimulated by different antigen fractions of *L. donovani* promastigotes prepared from the blotted nitrocellulose membrane as described above. After an incubation period of 96 h, [³H]-thymidine was added to the culture which was incubated for a further period of 20 h. Thereafter, cells were harvested and radioactive count was recorded as described above.
(e) Removal of adherence cells from the mononuclear cell preparation derived from the spleen of infected BALB/c mice

In some experiments, splenic mononuclear cell suspension (after separation by histopaque 1077 gradient) derived from *L. donovani* infected mice was rendered free from the adherent type cell population by the plastic plate adherence method (Gifawesen and Farrell, 1989). For this, plastic tissue culture dishes (25 mm diameter, Tarson Products, India) were pretreated with serum containing medium (RPMI 1640 with 15% FBS) for overnight at 37°C in 5% CO₂ atmosphere in a CO₂-incubator (Nuaire, USA) to improve their adherence properties. Next, freshly prepared splenic mononuclear cell suspension was taken in the plastic plates and incubated for about 2.5 h at 37°C in 5% CO₂ atmosphere in a CO₂-incubator. Following incubation, the nonadhering cells (primarily consisting of lymphocytes) were collected by using a siliconized pasteur pipette and centrifuged (300 x g) at 4°C. The cell pellet, thus obtained, was resuspended in RPMI 1640 medium (containing 15% FBS) and washed twice by centrifugation (300 x g) at 4°C. Finally, the cell pellet was resuspended in 1 ml of RPMI 1640 medium (containing 15% FBS). Number of cells in the suspension was counted by using a hemocytometer in a similar way as described in Section 2.1.2. Viability and purity of the cell suspension was also checked by the methods similar to those described earlier (vide Section 2.1.2).
2.12. Determination of the protective efficacies of different leishmanial antigen preparations in BALB/c mice against challenge with *L. donovani* amastigotes

Groups of age and sex matched BALB/c mice were immunized with different preparations of *L. donovani* promastigote antigens [e.g. heat-killed (HK), formalin-killed (FK) and sonicated-killed (SK) promastigotes] (vide Sections 2.7.1, 2.7.3 and 2.7.4 respectively) via the subcutaneous route. The immunization consisted of a dose of about $2 \times 10^7$ parasite equivalent material/0.1 ml/animal. Each group (about 6 animals per group) of animals received weekly one such immunization for three weeks and a fourth immunization after a fortnight interval (i.e., on day 0, 7, 14 and 28). Control group of animals received normal saline only. Immunized animals, including the controls, were challenged with *L. donovani* amastigotes ($-2 \times 10^7$ parasites/0.1 ml/animal) 7 days after the last immunization. Prior to amastigote challenge, immunized animals were bled intracardially or by the retro-orbital venus plexus and sera collected. Sample sera (about 0.1 ml) were decomplemented by heating at $56$ °C for 30 min in a water bath and kept at $-20$ °C for serological tests. All animals, including the control group, were sacrificed 30 days after the day of challenge and the degree of parasitemia in their liver was determined from the organ impression smears in a similar way as described in Section 2.3.2.
The efficacy of different leishmanial antigen preparations to induce cell-mediated immune responses in BALB/c mice was tested by lymphocyte transformation experiments in vitro. For this, splenic lymphocytes were isolated (vide Section 2.11 a), by sacrificing the HK, FK and SK immunised animals 7 days after the last immunization. Cells, thus obtained, were stimulated in vitro with leishmanial antigen (freeze-thawed) by incubating at 37°C for a total period of 116 h. Twenty hours prior to harvesting, cells were pulsed with \[^{3}H\]-thymidine and radioactivity incorporated was determined as described earlier (vide Section 2.11 b).

2.13. Determination of the effect of protein A (from Staphylococcus aureus) treatment (with or without antileishmanial drug sodium stibogluconate) of BALB/c mice infected with L. donovani

Groups of BALB/c mice were infected with L. donovani amastigotes (about 2 x 10^7 parasites/0.1 ml/animal) via intracardial route. Treatment of the infected animals started on day 8 following leishmanial infection. Different groups received different types of treatment as per the following protocol:
(i) Group I (6 mice) was treated with protein A (60 ug/kg body weight/dose) (prepared from the strain Staphylococcus aureus Cowan I, Pharmacia, Sweden) twice weekly for two weeks via the intraperitoneal route.
(ii) Group II (6 mice) was treated with the antileishmanial drug sodium stibogluconate [(stibanate, Gluconate, India, 20
mg SbV/kg body weight/dose)] twice weekly for two weeks via intraperitoneal route.

(iii) Group III (6 mice) was treated with a combination of protein A and stibanate via the same route. Each animal received four protein A injections [described under item (i) as above] and four stibanate injections [described under item (ii) as above] during the period of two weeks.

(iv) The control group (6 mice) received normal (0.85%) saline only during this period.

All animals, including the control group, were sacrificed 30 days after the day of infection and the degree of parasitemia in their liver was determined as described earlier (vide Section 2.3.2).