Immunogenic Potential of Cytosolic Proteins Against Visceral Leishmaniasis in BALB/C Mice and the Role of Chitosan as Adjuvant

KARAM DAWOOD SALMAN¹, SAIF QAHTAN SALMAN², GHUFRAN A. ABDURALHEEM³

¹Lecturer of Immunopathology. College of Veterinary Medicine, Al-Kufa University, Kufa, Iraq, E-mail: karam.biotech@mail.com.
²Lecturer of Biochemistry, College of Biotechnology, Al-Qasim Green University, Babylon, Iraq, E-mail: saif.q1988@gmail.com.
³Lecturer of Zoology, College of Biotechnology, Al-Qasim Green University, Babylon, Iraq, E-mail: gogo868776@yahoo.com.

Abstract: Isolation of the cytosolic antigens (CytAgs), its immune response study and proteome profiling is an essential prerequisite for understanding the molecular pathogenesis of Leishmania donovani and immunogenic potential of these proteins. The immunestimulatory potential of L. donovani CytAgs, purified from culture of L. donovani clinical isolate was evaluated for their ability to induce cellular responses in treated/cured BALB/c mice. This study done by evaluation of humoral immunity in terms of IgG and IgG isotypes production which in turn correlates with potentiation of Th1 (cell mediated immunity) and Th2 (humoral immune response) type responses. As Cytosolic with chitosan immunized group elicited similar IgG and IgG isotype response in comparison to cytosolic alone immunized group. The higher levels of IgG1 after 10 days immunization demonstrates that a concomitant Th2 response with L. donovani cytosolic protein doesn’t inhibit a strong Th1 effector function. The antigenicity of cytosolic protein components were demonstrated by means of western blotting. Immunoblot profile was established followed by reaction with unimmunized and immunized mice sera from different groups. Additional reactivity was observed in cytosolic and chitosan immunized group with bands 96 and 48 kDa.

Keywords: Leishmania Donovani, IgG, Chitosan, Western Blotting.

I. INTRODUCTION

Visceral leishmaniasis (VL), also known as kala-azar, is a disseminated protozoan infection caused by Leishmania donovani complex (Murray et al., 2005). VL is essentially caused by L. donovani and Leishmania infantum (synonym Leishmania chagasiin South-America). Exceptionally, visceralization of species typically associated with cutaneous leishmaniasis has been observed. Most commonly, this has been reported with Leishmania tropical in the Middle East and Leishmania amazonensis in South-America. In individuals infected with human immuno deficiency virus (HIV), visceralization of a number of dermatotropic species has been documented as well (see section on VL-HIV coinfection). Current treatment for VL based on chemotherapy, which relies on a handful of drugs with serious limitations such as high cost and toxicity, difficult route of administration and lack of efficacy in endemic areas. The pentavalentantimonials such as sodium stibogluconate and meglumine antimoniate have been recommended for the treatment of leishmaniasis for over 70 years. It is thus not surprising that resistance to this class of drug is increasing, and in some endemic areas their use is limited due to a lack of efficacy. Second line drugs used in the treatment of leishmaniasis include aromatic diamidines (Pentamidine) and amphotericin B, but like to the pentavalentantimonials, these drugs are also toxic, with severe(sometimes life-threatening) side effects (Kedzierskiet al., 2009).

A development of a successful vaccine to prevent leishmaniasis has been a goal for almost a century, but currently no such vaccine exists. Extensive evidence from studies in animal models, mainly mice, indicates that solid protection can be achieved upon immunization with defined subunit vaccines (either protein or DNA) or heat-killed parasites, however, to date such vaccines have been disappointing when tested in field studies (Kedzierskiet al., 2006). First attempts at vaccination, termed leishmanization, were based on the observation that following lesion healing an individual is refractory to reinfection. Initially, infectious lesion material, later replaced by cultured parasite inoculum, has been used to inoculate uninfected individuals. This method has been largely discontinued due to a range of reasons including quality control, parasite persistence, emergence of HIV and ethical reasons, amongst the others. The first-generation vaccines based on killed parasites have replaced leishmanization, but this type of vaccines have shown poor efficacy in clinical trials (Noazin et al., 2008). Leishmania vaccine development has proven to be a difficult and challenging task, which is mostly hampered by inadequate knowledge of parasite pathogenesis and the complexity of immune responses needed for protection.

These aspects are of key importance in the vaccine development process. Vaccination against VL has received limited attention compared with cutaneous leishmaniasis (CL). Historically CL has been the focus of vaccination attempts, as it has been known for centuries that people who resolve a primary CL skin lesion are protected from further infections. It is generally acknowledged that human VL
trials will follow on from any successful CL immunization programme. Ideally a vaccine would provide cross-protection against multiple Leishmania species. The recent comparative genomic analysis of three Leishmania species, which cause distinct disease pathologies, showed that L. major, L. braziliensis, and L. infantum genomes are highly conserved and have very few species-specific genes (Peacock et al., 2007). There is a high degree of variability in the cross-protective immunity induced by infection with different Leishmania species (Porrozziet al., 2004) and VL-specific vaccines may provide a more successful intervention.

II. MATERIALS AND METHODS
A. MICE AND PARASITES
BALB/c mice, bred in the animal care facility of the University under pathogen-free conditions, were used at 4 to 6 weeks old for experimental purposes with prior approval from the Animal Ethics Committee of the Jamia Hamdard. An Indian strain of L. donovani AG83 was maintained by passage in BALB/c mice, amastigotes isolated from infected mice spleens were allowed to transform to promastigotes by cultivation at 22°C in M199 medium (pH 7.4) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml of penicillin G-sodium, and 100 µg/ml of streptomycin sulfate. Parasites were checked and enumerated by counting in a hemocytometer on day 6 after first transformation and every 72 hrs thereafter. Designated parasites from stationary-phase cultures were diluted in fresh medium with the same composition as that mentioned above to maintain an average density of 2 x106 cells/ml (Afrin et al, 2002).

B. Determination Of Antigen Concentration By Lowry’s Method
Principle: The “Lowry Assay: Protein by Folin’s Reagent” (Lowry et al., 1951) has been the most widely used method to estimate the amount of protein (already in solution or easily-soluble in dilute alkali) in biological samples. First the proteins are pretreated with copper ion in alkaline solution, and then the aromatic amino acids in the treated sample reduce the phosphomolybdate-phosphotungstic acid present in the Folin’s Reagent. The end product of this reaction has a blue color. The amount of protein in the sample can be estimated via reading the absorbance (at 750 nm) of the end product of the Folin reaction against a standard curve of a selected protein solution (in our case; Bovine Serum Albumin (BSA) solution).

The Lowry Method Relies On Two Different Reactions:
- The first reaction is the formation of a copper ion complex with amide bonds, forming reduced copper in alkaline solutions. This is called a Biuret chromophore and is commonly stabilized by the addition of tartarate (Gornall et al., 1949).
- The second reaction is reduction of the Folin-Ciocalteau reagent (phosphomolybdate and phosphotungstic acid), primarily by the reduced copper-amide bond complex as well as by tyrosine and tryptophan residues.

C. Preparing the Solution
- The Lowry solution is a mixture of the (Na2 Tartrant.2(H2O), CuSO4.5(H2O) and Other NaOH, Na2CO3) chemicals, except the Folin’s Reagent. The Lowry solution should be prepared fresh, at the day of measurement. Though the individual solutions for the Lowry solution can be prepared in advance and then mixed at the day of measurement.
- Solution A is a dilute alkali solution. 2N Folin and Ciocalteau’s Phenol Reagent contain HCl and H2PO4.

Solution A: (alkaline Solution) (for 100 ml)
0.572gm NaOH
2.862gm Na2CO3

Solution B: (for 20 ml)
0.285gm CuSO4.5(H2O)

Solution C: (for 20 ml)
0.571gm Na2Tartrant.2(H2O)

Lowry Solution: (freshly prepared, 0.7/ml sample)

Folin Reagent: (instant fresh, 0.1 ml/sample)

5ml of 2N Folin and Ciocalteu’s Phenol Reagent + 5ml of double distilled water this solution is light sensitive. So it should be prepared at least 5min of the first sample incubation and kept in an amber container.

Procedure:
- Different dilutions of BSA are prepared by mixing stock BSA (1 mg/ml) and water in the test tube. Similarly, two dilutions of antigen are prepared in duplicate. The final volume in each of the test tubes is 5 ml. The BSA concentration ranges from 0.05 to 1 mg/ml.
- From these different dilutions, pipette out 0.2 ml BSA or antigen to different test tubes and add 2 ml of alkaline copper sulphate reagent. Mix the solutions well.
- This solution is incubated at room temperature for 10 mins.
- Then add 0.2 ml of reagent FolinCiocalteau to each tube and incubate for 30 min. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660 nm.
- Plot the absorbance against BSA concentration to get a standard calibration curve.
- Check the absorbance of antigen and determine the concentration of the unknown sample using the standard curve.

D. SDS-PAGE
Principle: Electrophoresis is the study of the movement of charged molecules in an electric field. The generally used
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Support medium is cellulose or thin gels made up of either polyacrylamide or agarose. Cellulose is used as support medium for low molecular weight bio-chemicals such as amino acid and carbohydrates whereas agarose and polyacrylamide gels are widely used for larger molecules like proteins. The general electrophoresis techniques cannot be used to measure the molecular weight of the biological molecules because the mobility of a substance in the gel is influenced by both charge and size. In order to overcome this, if the biological samples are treated so that they have a uniform charge, electrophoretic mobility then depends primarily on size. The molecular weight of protein maybe estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS) and a reducing agent mercaptoethanol (ME). SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. 1.4grams of SDS binds per gram of protein. Mercaptoethanol assists the protein denaturation by reducing all disulfide bonds.

E. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels are prepared by the free radical polymerization of acrylamide and the cross linking agent N N’ methylene bis acrylamide.

Preparation of A 10% Resolving/Separating Gel:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide + 0.8% Bisacrylamide</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>4X Tris-HCl/SDS, pH 8.8</td>
<td>0.94 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.56 ml</td>
</tr>
<tr>
<td>10% ammonium persulfate (APS)</td>
<td>25 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Preparation of A 4.5% Stacking Gel:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide + 0.8% Bisacrylamide</td>
<td>0.65 ml</td>
</tr>
<tr>
<td>4X Tris-HCl/SDS, pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>25 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

The Leishmanial antigen (Cytosolic) from L. donovani-promastigotes was subjected to sodium dodecyl sulphate 10% polyacrylamide gel electrophoresis (SDS–10% PAGE), and the proteinswere localized in gels, stained with Coomassie blue. (Ulrich K. Laemmli, 1970).

F. Silver Staining

Principle: Silver staining is the most sensitive method for permanent staining of proteins in polyacrylamide gels. It creates a record of the electrophoresis result that can be viewed without any special equipment. It is, however, a complex, multi-steps process, and many variables can influence the result. High purity reagents and precise timing are necessary for reproducible, high-quality results. In silver staining, polyacrylamide gels are impregnated with soluble silver ion (Ag+) and developed by treatment with a reductant. Macromolecules in the gel promote the reduction of silver ion to metallic silver (Ag0), which is insoluble and visible, allowing protein- or nucleic acid-containing bands to be seen. The initial deposition of metallic silver promotes further deposition in an autocatalytic process, resulting in exceptionally high sensitivity.

Steps in The Silver Staining Process:

The silver staining process consists of the following steps: Fixing, sensitization, silver impregnation, development, stopping, and gel preservation. Water washes are also included between some of the steps.

Fixing: In the fixing step, the gel is treated with acid. This renders the macromolecules in the gel insoluble and prevents them from diffusing out of the gel during subsequent staining steps. Substances in the gel that interfere with silver staining such as buffers, ions, denaturants, detergents or carrier ampholytes, are washed out of the gel during this step.

Sensitization: The gel is treated with reagents that chemically modify proteins, rendering them more reactive toward silver, and reagents that accelerate the subsequent reduction of silver ion. This step greatly enhances the sensitivity of silver staining for protein. Excess sensitization reagent results in a high level of background staining, so the...
gel is washed thoroughly with distilled or de-ionized water following the sensitization step.

**Silver Impregnation:** In this step, the gel is treated with silver nitrate. Mildly acidic conditions prevent silver ion from being reduced to metallic silver. The gel is briefly washed following this step to remove excess silver from the gel surface.

**Development:** The development solution contains formaldehyde, which reduces silver ion to metallic silver. This reaction only proceeds at high pH, so sodium carbonate is included to render the development solution alkaline.

**Stopping And Preservation:** The stopping solution prevents further reduction of silver ion. The preservation solution contains glycerol, which prevents the gel from cracking during drying.

**Procedure:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Conc.</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fix</td>
<td>50% methanol, 10% acetic acid</td>
<td>Can be held overnight</td>
<td>30 min</td>
</tr>
<tr>
<td>Fix</td>
<td>50% methanol</td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>Milli-Q water</td>
<td></td>
<td>5 ± 1 min</td>
</tr>
<tr>
<td>Sensitive</td>
<td>Sodium thiosulfate</td>
<td>0.2 g/L</td>
<td>1 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>Milli-Q water</td>
<td></td>
<td>2 ± 1 min</td>
</tr>
<tr>
<td>Silver</td>
<td>Silver nitrate (chilled to 4°C)</td>
<td>2 g/L</td>
<td>25 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>Milli-Q water</td>
<td></td>
<td>2 ± 1 min</td>
</tr>
<tr>
<td>Develop</td>
<td>Sodium carbonate anhydrous 0.025% Formalin</td>
<td>30 g/L, 250 mL/L</td>
<td>1. Incubate until solution turns yellow; 2. Develop 5 min or until desired stain intensity</td>
</tr>
<tr>
<td>Stop</td>
<td>EDTA, sodium salt</td>
<td>14 g/L</td>
<td>10 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>Milli-Q water</td>
<td></td>
<td>2 ± 1 min</td>
</tr>
</tbody>
</table>

(Morrisey, 1981).

**G. Immunization of Mice**

4 to 6 weeks old BALB/c mice were divided into 4 groups (4 mice per group). The first group was administered 1XPBS subcutaneously (s/c) and was taken as control. Second group was immunized with 100 μg/200 μl of cytosolic antigen (three s/c injections, 14 days intervals) phosphate-buffered saline (PBS). Third group of animals received cytosolic antigen along with chitosan and fourth group was injected with chitosan alone. At 10 days after 3rd immunization, mice were bleed and serum collected from all four groups and then the mice were sacrificed for immunological assays. (Afrin et al., 1990).

**III. RESULTS**

**A. Determination of Antigen (Cytosolic) Concentrations by Lowry's Method**

The concentration of cytosolic antigen was determined by Lowery’s method using BSA as a control to generate standard curve (Fig.2) and the concentration of cytosolic antigen was found to be 3.32 mg/ml.

**Fig.2.** BSA standard graph for estimation of protein concentration.

**Fig.3.** SDS profiling of cytosolic proteins in comparative with ladder.

4 to 6 weeks old BALB/c mice were divided to 4 groups (4 mice per group). The first group was administered 1XPBS subcutaneously (s/c) and was taken as control. Second group was immunized with 100 μg/200 μl of cytosolic antigen (three s/c injections, 14 days intervals) phosphate-buffered saline (PBS). Third group of animals received cytosolic antigen along with chitosan and fourth group was injected with chitosan alone. At 10 days after 3rd immunization, mice were bleed and serum collected from all four groups and then the mice were sacrificed for immunological assays. (Afrin et al., 1990).
B. Characterization Of Antigen (SDS-PAGE)

The protein profile was examined by SDS-PAGE. SDS-PAGE analysis revealed that cytosolic antigen is a mixture of polypeptides with molecular mass ranging from 27 to 96KDa. The prominent polypeptides correspond to molecular weight 48 KDa and 38 KDa (Fig.3).

C. Total IgG by ELISA

To investigate the induction of humoral immune responses, mice (immunized and normal or PBS injected) sera were assayed for leishmanial Ag specific IgG level through ELISA. The Ag stimulated a substantial IgG response as visible from the graph and also Ag along with adjuvant stimulated comparable level of IgG (Fig.4).

![Fig.4. Total IgG detection by ELISA.](image)

D. IgG subtypes by ELISA

Leishmania-specific IgG isotypes analysis showed strong levels of IgG1 antibody in the immunized mice in comparison to IgG2a, IgG2b, and IgG3 antibodies after 10 days post immunization. Maximum level of IgG1 was induced in cytosolic antigen with adjuvant group followed by cytosolic alone immunized group. The concomitant stimulation of the IgG2a, and IgG3 isotyep although to a lesser extent demonstrates the induction of a mixed Th1 and Th2 response after immunization with cytosolic + adjuvant group. Further, the levels of isotypes elicited post immunization with chitosan alone and chitosan along with adjuvant were comparable in IgG2a, IgG 2b and as well as in IgG3 demonstrating the immunogenic capacity of chitosan antigen alone (Fig.5).

E. Western Blotting Analysis

The antigenicity of cytosolic antigen in sera from mice immunized with cytosolic antigen was studied by performing western blot analysis. Cytosolic antigen was separated by SDS-PAGE and the proteins were immunoblotted with sera from mice after immunization with cytosolic antigen. A similar blot of cytosolic antigen was also probed with sera from unimmunized mice and mice that received chitosan alone (data not shown). Antigenic profile was characterized by a dominant 38kDa and 48 kDa bands in cytosolic antigen (Fig.20). The antibodies with enhanced titres that were elicited after immunization of mice with cytosolic antigen along with chitosan recognized several antigens of cytosolic protein with protein bands (48, 64 and 96 kDa) (Fig.21). Antigen recognition by immunized mouse sera after 10 days post immunization demonstrated a reactivity predominantly for these moieties.

![Fig.6.](image)

IV. CONCLUSION

Leishmania-specific IgG isotypes analysis showed strong levels of IgG1 antibody in the immunized mice in comparison to IgG2a, IgG2b, and IgG3 antibodies after 10 days post immunization. Maximum level of IgG1 was induced in cytosolic antigen with adjuvant group followed by cytosolic alone immunized group. The concomitant stimulation of the IgG2a, and IgG3 isotypes although to a lesser extent demonstrates the induction of a mixed Th1 and Th2 response after immunization with cytosolic + adjuvant group. Further, the levels of isotypes elicited post immunization with chitosan alone and chitosan along with adjuvant were comparable in IgG2a, IgG 2b and as well as
in IgG3 demonstrating the immunogenic capacity of chitosan antigen alone. Antigenic profile was characterized by a dominant 38kDa and 48 kDa bands in cytosolic antigen. The antibodies with enhanced titres that were elicited after immunization of mice with cytosolic antigen along with chitosan recognized several antigens of cytosolic protein with protein bands (48, 64 and 96 kDa). Differential western blot profile along with above mentioned findings indicates the immunogenic potential of cytosolic protein. Primary evidences exhibit that immunization of cytosolic protein in association with chitosan can enhance the immune response in BALB/c mice through subcutaneous route. These preliminary findings thus, advocate further evaluation of therapeutic efficacy of this protein formulation against visceral leishmaniasis infection in BALB/c mice.

V. REFERENCES