International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

A new experimental survey for immunogenicity evaluating of GP_{63} purified from *Leishmania major in BALB/c* mice

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Abstract

Leishmaniasis is a major health problem in some foci of 88 endemic countries. The major surface glycoprotein $_{63}(GP_{63})$ is a membrane-bound that participates in attachment of the promastigotes (PM) stage of this protozoan to host macrophages. The aim of present study was a new experimental survey for immunogenicity evaluating of gp_{63} Purified from Leishmania (Leishmania) major in BALB/c mice. Three groups' control group: did not receive adjuvant and gp63, adjuvant group: only received adjutant and gp_{63} group: recipient gp_{63} and adjuvant of 10 BALB/c mice. *Leishmania (L) major [MRHO/IR/75/ER]* strains PMs. It was proliferated in NNN and RPMI1640 with high density the PMs (10^{10}Cells) were harvested in late exponential phase by (2000×g.15min) then, PMs by using affinity chromatography on Con-A conjugated Sepharose 5B for GP_{63} was purified and role of immunity was evaluated separately one by one and mixed with Frond's complete and incomplete adjutants on BALB/c mice. The mean weight of the mice and the mean diameter of the lesions within the groups showed a statistically significant difference. (P=0.04). The mean weight of control groups no statistically significant difference with case and adjuvant groups (P= 0.07). The mean diameter of the lesions controls group was significant difference statistically adjuvant and case groups. The results showed decrease of weight and injury development on mice which received GP_{63}.

Keywords: *Leishmania (L) major*, Purification, GP63, in vivo, Experimental survey.

Introduction

Leishmaniae are obligatory intracellular protozoa in human mononuclear phagocytes. They cause a spectrum of diseases, ranging in severity from spontaneously healing skin lesions to fatal visceral disease. The disease is a significant health problem in many parts of the world resulting in an estimated 12 million new cases each year *Leishmania* are protozoan parasites spread by a sand fly insect vector and causing a spectrum of diseases collectively known as leishmaniasis. The current high cost of drug resistance and toxic side effects, it is necessary to identify and develop alternative therapies.Worldwide, there are 2 million new cases each year and 1/10 of the world's population is at risk of infection. To date, there are no vaccines against leishmaniasis and control measures rely on chemotherapy to alleviate disease and on vector control to reduce transmission (1). The GP_{63} is a membrane-bound. Most studies show that the enzyme neutral or acidic (2). Glycolipid- anchored zinc protease is on surface of All *Leishmania* species, called GP_{63}. Gp63 participates in attachment of the promastigotes stage of these protozoan parasites to host macrophages. In vitro, most Leishmania species are non-pathogenic form logarithmic into a statistic virulent form (**3**). This molecule is well conserved among Leishmania species that includes amino acid

sequences between 255-252 SRYD. Also a lot of fibronectin antibodies have been shown to cross-react with the binding of promastigotes to macrophages and prevents (4). The role of GP_{63} for *Leishmania* in the vector is less clear. It has been suggested that GP_{63} may degrade hemoglobin and other proteins in the blood meals, thereby providing nutrients needed for the growth of promastigotes.this and additional functions may be further suggested by the finding of similar ectoproteases in other trypanosomatids.in contrast, GP_{63} is thought to play little or no role for *Leishmania(L).major* in the vectors, since knockout mutant were found to develop and survive as well as the wild type in three old world Phlebotomus species(5, 6 and 7). The aim of present study was a new experimental survey for immunogenicity evaluating of the major surface glycoprotein of *Leishmania* GP_{63} purified from strain of *Leishmania (Leishmania) major [MRHO/IR/75/ER]* in BALB/c mice.

Materials and Methods

1. Parasite cultivation

Leishmania(L) major [MRHO/IR/75/ER] that isolated from lesion- derived amastigotes BALB/c infected, cultured in NNN media(Novy-Macnal-Nicol) and later were transferred to the $RPMI_{1640}$ medium enriched with 20% FCS, 2 mM L-glutamine, 200 U/ml Penicillin and 200 µg/ml Streptomycin (Sigma st.Louis.MQ), And then promastigotes (10^{10}Cells) were harvested in late exponential Phase by centrifugation (2000×g.15min).(Fig.1).

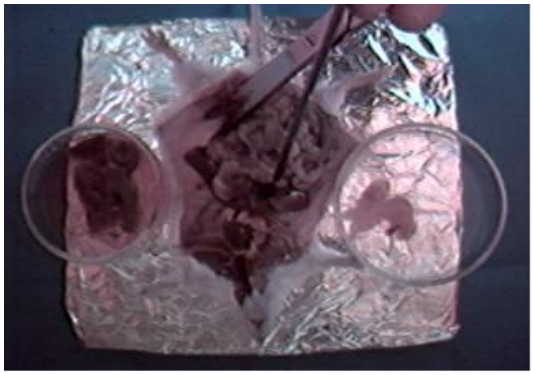

Fig1. *Leishmania (L) major [MRHO/IR/75/ER]* that isolated from lesion- derived amastogote BALB/c infected.

2. Purification of *Leishmania (L) major [MRHO/IR/75/ER]* Gp_{63} pelleted fold

Promastigotes (10^{10}Cells) were frozen and thawed in50mM Tris, PH7.5and sonicated on ice at 45 W for3×10s to use cells. Peripheral membrane proteins were dissociated using 3M Nacl in lyses buffer followed by centrifugation at 20,000×g for 20min.After three repetitions, the integral membrane proteins were extracted by stirring for 24 h at4c in 1% octylthioglucoside (sigma).the resulting extract was diluted five ford with buffer A(20mM tri,PH7.2)1mM each of $cacl_2$,$mgcl_2$ and $Mncl_2$,and 0.1% chaps(sigma)and applied to a concavalin A(con-A) Sepharose (pharmacia,baied,urfe,quebec)at a flow rate of 0.2 ml min^{-1}.the column was washed with 2 bed volumes of buffer A to remove non-specifically bound protein followed by elution with 3 bed volumes of buffer B (buffer a with 500 Mm methylmannopyranoside and 400 mM Nacl) to elute bound ligands.The con-A elute was dialysed,lyophilized and resuspended in SDS,10% glycerol,0.2% ß-mercaptoethanol) and the proteins separated using a model 230 A micro preparative electrophoresis (Applied Biosystem,Foster city,CA).Electrophoresis was performed with constant current over a voltage range of 180_260 V using a 7.5% polyacrylamide gel(2.5 × 50mm) Proteins were monitored at 280 nm at a sensitivity of 0.5 AUFS. The upper buffer consisted of 25mM Tris_HCI, 192mM glycine, 0.1% SDS, while the lower and elution buffers were 25mM Tris_HCI (pH 8.3). Fractions were assessed for purity by SDS_PAGE using a 12.5% gel. The purified GP_{63} was dialyzed, lyophilized and SDS was extracted by washing the Precipitate three times with cold acetone/water (20:1v/v) alliwing20 min incubation on dry Ice to maximize precipitation of protein. A final wash with 100% acetone facilitated drying of the sample (8 9, 10, 11, and 12).

3. Identification of GP_{63} Molecule

Dot-ELISA, the nitrocellulose membranes were sensitized with drops containing 1µ g of antigen purified by affinity chromatography. Once sensitized and blocked, they were exposed for 30 min to the serum samples (1: 250) and then treated with peroxidaseconjugated anti-human (1: 1600) or anti-canine (1: 1200) immunoglobulin (Sigma) for the human and canine samples, respectively.

O-phenylenediamine dihydrochloride (Sigma) and 10µ L of 30% H2O2 per 25 mL were finally used to detect the presence of. The membranes were scanned and processed by Quantity Scan version 1·25.

4. Vaccination of BALB/c mice against GP63 molecule from *Leishmania (L) major*.

Three Groups [Control, Adjuvant and Adjuvant groups of 10 BALB/c mice (ten mice in each group)]were the study groups and experiments Vaccination of BALB/c mice against of GP_{63} Molecule. **a.** Control group: Did not receive adjuvant and GP_{63}. **b.** Adjuvant group: only received adjutant. **c.** GP_{63} group: Recipient GP_{63} and adjuvant. Mice in all groups were in Week 8 although the weight of all animals was not the same. Promastigotes of *Leishmania (L.major,* strain[*MRHO/IR/75/ER*]were grown at 26°C in a semi-defined $RPMI_{1640}$ medium (Gibco) containing 25 mM Hepes, pH 7.4, 2 mM glutamine and 10% heat-inactivated fetal calf serum. Streptomycin at 5 pg/ml, penicillin at 5 U/ml and kanamycin at 5pg/ml were also added. Promastigotes were harvested after four times passages in the stationary (Fig.2).

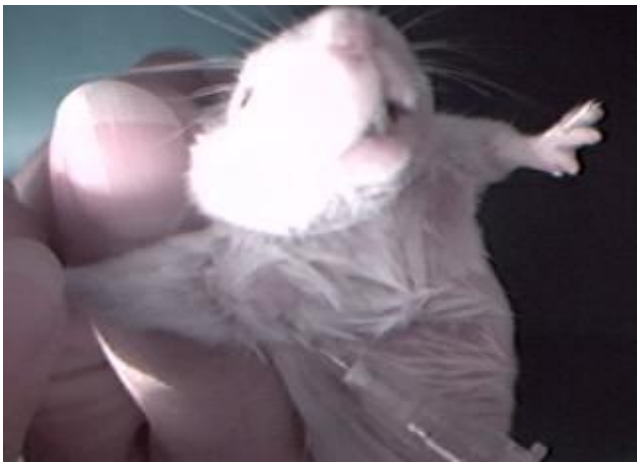

Fig 2. Vaccination of BALB/c mice against of GP_{63} Molecule.

Phase of growth, and then BALB/c mice (ten mice in each group)
a. Control group: Did not receive adjuvant and GP_{63}.
b. Adjuvant group: only received adjutant. c. GP_{63} group: Recipient GP_{63} and.

Were infected with 1 X 10' *Leishmania (L).major[MRHO/IR/75/ER*] promastigotes by tail-vein injection of a 0.1-ml in oculum. Infection was assessed 6 weeks later by comparing parasite load of the liver. Impression smears, prepared from a cut surface, were fixed with methanol and stained with Giemsa. The number of amastigotes500 liver cell nuclei was determined microscopically. Mice were monitored for weight and diameter wound up dead last of all groups of mice in the control group continued.

5. Statistical analysis

Data are expressed as Mean ± SEM of lesion's diameter and animal's weight, and were statistically analyzed by multivariate test (Hotelling's Trace) for analyzing the effect of time within-subjects and multiple comparisons test (Tukey HSD) for comparing the variables between groups. In this study, $p < 0.05$ was considered as the significant level.

Results and Discussion

1. Using Column of ConA - conjugated sepharos 5B affinity chromatography method is very specific, almost all 500000 copies GP_{63} molecules leptomonades membrane separates from the purity of the Proof PAGE and SDS - PAGE , then for showing out to be positive, Dot - ELISA performed (Fig. 3)

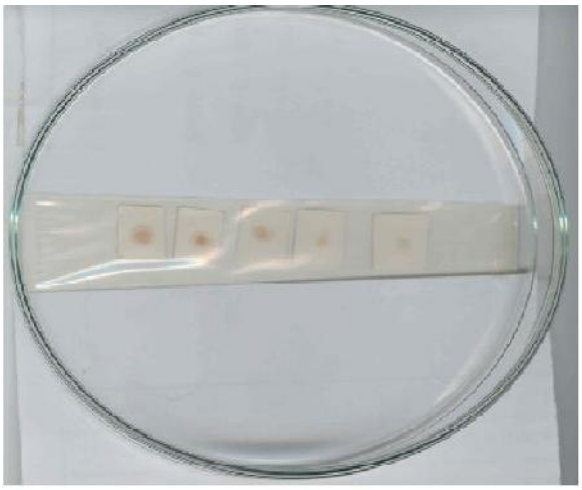

Fig3. Dot - ELISA GP63, Concentration increases from left to right.

2. Evaluation of the immune response against GP_{63}, purified from Leishmania *(L) Major [MRHO/IR/75/ER*] on BALB /c mice.

2- 1-Mean and SD of weight of BALB/c mice Groups:

Based on repeat measure the statistical method and Hotelling's Trace multivariate Test, time and the mean weight of in all groups showed statistically significant differences.($p < 0.00001$). And the mean weight of the mice within the groups showed a statistically significant difference. ($p=0.04$). According to the HSD Tukey test for multiple comparisons, the mean weight of control group statistically no significant difference with case and adjuvant groups. ($p=0.07$). (Figure.4).

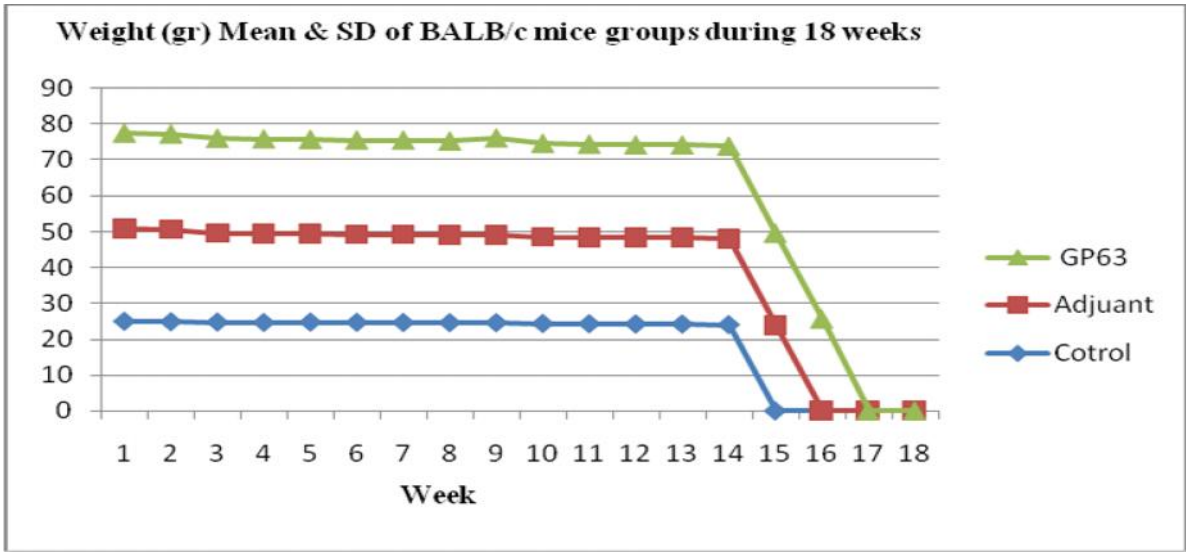

Fig 4. Weight (gr) Mean & SD of BALB/c mice groups during 18 weeks. HSD Tukey test for multiple comparisons (P=0.07)

2. 2. Diameter of sore (mm) Mean & SD on BALB/c mice ggroups during 18 weeks.

Based on repeat measure the statistical method and Hotelling's Trace multivariate test of time and the mean diameter of the lesions in all groups showed statistically significant differences.(p <0.00001). And the mean diameter of the lesions within the groups showed a statistically significant difference. (P=0.04). According to the HSD Tukey test for multiple comparisons, controls group no significant difference with statistically adjuvant group. (P=0.11) but between control and case group is statistically significant difference. (P=0.03). (Figure 5.).

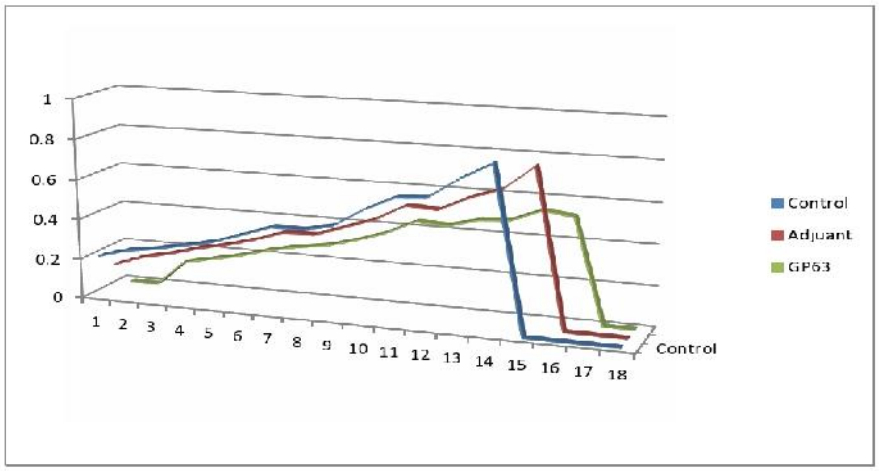

Fig 5. Diameter sore **(mm)** Mean & SD on BALB/c mice ggroups during 18 weeks. HSD Tukey test for multiple comparisons (P=0.03)

Weight loss and increased lesion diameter in the control group of mice was higher than all groups and Weight loss and increased lesion diameter in the case group of mice was lowest. (Fig.6-8).

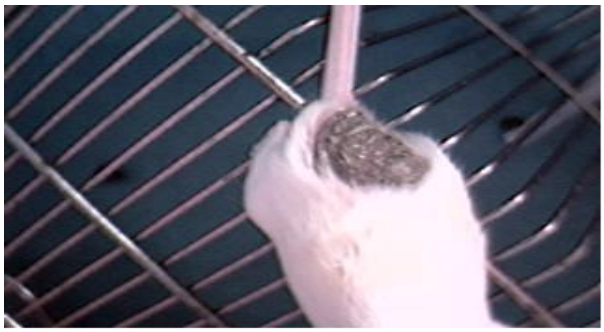

Fig6. Control group, sore diameter in the fiftteenth Week.

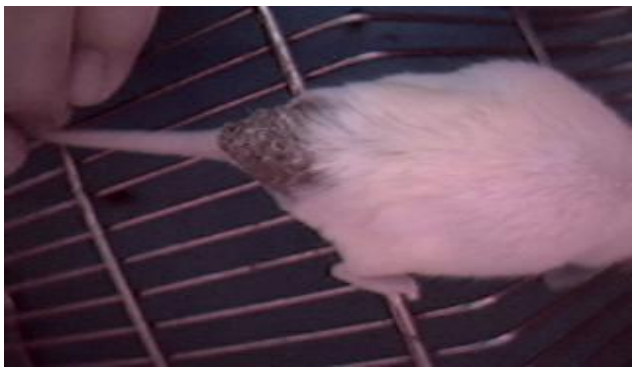

Fig7. Adjuvant group, sore diameter in the fifteenth Week

Fig8. GP_{63}+ Adjuvant group, sore diameter in the fifteenth Week

2.3. Promastigotes number and diameter (mm) of each BALB/c liver

Promastigotes in the control group had the highest number and the lowest number GP63 and so The maximum diameter of the liver in the control group and was lowest in Group GP63. The present investigation aimed a new experimental survey for immunogenicity evaluating of the major surface glycoprotein purified from *Leishmania (Leishmania) major [MRHO/IR/75/ER]* in BALB/c mice. Previous studies showed that GP_{63} was a serologically specific marker of human leishmaniasis. The efficacy of a vaccine formulation relies not only in the specificity of its antigen but also on the ability of the adjuvant to trigger an efficient protective response. For an immunosuppressor intracellular parasite like *L. donovani*, a good adjuvant should guarantee the maintenance of a strong humeral and cellular immune response against the pathogen (13). Air pollution, global warming, human development and other areas of development of the disease has increased in recent decade's spectrum of reports across the world, no vaccine, drug and Re-emerging disease in many geographic areas except in some areas of the emerging diseases. It is important that the focus of most health professionals is infectious and non-sterile immunity in this disease. Debilitating diseases such as AIDS the immune system is back and relapse in patients with AIDS and leishmaniasis of potentially dangerous. In the past decades, studies and research on leishmaniasis vaccines and drugs has been widespread and abundant information about the activities carried out during the past few decades has been published. During growth, the promastigotes form of *Leishmania* protozoa in the NNN and $RPMI_{1964}$ mediums, develops from a less infectious form during logarithmic (termed the procyclic promastigotes) growth to a highly virulent form (termed the metacyclic promastigote) at stationary phase. Accompanying this change in virulence are changes in parasite morphology (14 and 15) increased resistance to complement- and H_2o_2-mediated killing, lower respiratory rate , increased glycosylation of the surface lipophosphoglycan, an increase in hsp70 mRNA, increased expression of a protein containing a basic zipper motif, and an increase in the amount of gp_{63} protein. In the group of mice that were negative and did not receive any adjuvant or molecules as expected and natural healing process will ultimately lead to animal death. In the group of mice that receiving adjuvant beginning nodule incidence of severe reactions at the site of the scar, but the opening of the disease was similar in mice of negative drag by macrophages at the site of inoculation. Adjuvant severe reaction is like the certain antigens absent during the normal course of the disease is In the group receiving adjuvant GP_{63} process of weight loss, but slow and had the wound and eventually led to the death of the animal. In other words, although the molecular immunogenic and the immune response is not sufficient, however, spreads disease and death do not mix.The BALB/c mice infected to *Leishmania (L) major [MRHO/IR/75/ER*] and untreated caused a cutaneous leishmaniasis then vertebrate to visceral leishmaniasis and gradually decrease diameter of the sore, lose weight in animals, then infected animal dead. More Previous studies conducted with gp_{63} have shown that the majority of patients with visceral leishmaniasis from Brazil and Sudan had high levels of a bog GP_{63}, whereas patients with other forms of Leishmania showed no significant levels of specific Antibodies as determined by ELISA (16).

The protective efficacy of purified gp_{63} has been tested in several experimental models using different strains and adjuvant, giving rise to conflicting results. A small scale vaccine study of GP_{63} against *L. major* infection was performed in velvet monkeys. Three doses of the recombinant antigen were administrated mixed with BCG as an adjuvant. After vaccination, peripheral blood mononuclear cells (PBMC) from these animals neither proliferated nor produced IFN-g following stimulation with antigen, and only partial protection was achieved after challenge with virulent *L. major* promastigotes (17). Despite several decades of global efforts, few preparations of first generation *Leishmania* vaccine have reached phase 3 clinical trials.

Conclusion

The results of phase clinical trials showed an acceptable level of safety, but only limited efficacy was seen and no further improvement in efficacy was shown with multiple injections. The main reasons for this failure may be the lack of an appropriate adjuvant, require the presence of other surface molecules such as lipophosphoglycan (LPG), and require the presence of excretion – secretion antigens and so on. Therefore, it is concluded that co-encapsulation of GP_{63}, LPG, excretion – secretion antigens and appropriate adjuvant might be an appropriate strategy to induce a more potent Th1 immune response and improve protection against Leishmaniasis.

Acknowledgments

The authors give thanks to all people who have assisted the experimental procedure, especially to Mrs. Mahin ghafourzadeh Msc Medical Parasitology student, Marziyeh beigom modarres sanavi expert of laboratory technology department and to the research deputy of Yazd Shahid Sadoughi University of medical sciences, the sponsor of this research for their help and suggestions.

Authors' Contribution

All authors contributed equally to this work.

Financial Disclosure

This work was supported by grant No: 87-4153 from The Yazd Shahid Sadoughi University of medical sciences, Yazd, Iran for Dr. Ali Fattahi Bafghi's Project.

Funding/Support

There is no funding

Conflict of interest

None declared.

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