observed in group injected I/D by gold coated plasmid (50.90 pg/ml and 41.88 pg/ml respectively). The results also demonstrated that I/D injection of gold particles coated DNA vaccine induced the highest Th_1/Th_2 response [IL-2/IL-10 (4.348) and INF-γ/IL-4 (33.383)], while I/M injection of naked vaccine gave the lowest results [IL-2/IL-10 (1.951) and INF-γ/IL-4 (13.879)].

INTRODUCTION

Genetic immunization is a relatively new tool for achieving specific immune activation with several advantages such as expression of concerned genes nearest to its native form, induction of cellular immune response, persistent expression of desired antigen (Ag) and induction of memory responses against the infectious disease [(1)]. Moreover, host cells take up coding plasmids, transcribe and translate the encoded gene, and produce protein that stimulates an immune response when presented to the immune system in the context of self-MHC [(2)]. Notably, vaccination with plasmid DNA has been shown to induce protective immunity through both MHC class I- and class II-restricted T cell responses in a variety of infections [(3)]. Therefore, the plasmid DNA encoding specific Ag induced both $CD4^+$ and $CD8^+$ T cells, which is essential for protection against intracellular diseases that require cell mediated immunity like leishmaniasis [(4)].

DNA vaccines may provide better protection against *Leishmania* than killed or live-attenuated vaccines as they can induce the expression of *Leishmania* antigens, which are unaltered in their protein structure and antigenicity. Furthermore, bacteria-derived DNA plasmids are naturally immunogenic as their backbones contain unmethylated cytosine-phosphate-guanosine (CpG) motifs which have been shown to readily induce Th_1 cytokine expression and enhance $CD8^+$ T cell responses [(5)].

However, at present, there is no effective vaccine available anywhere in the world for routine use against leishmaniasis[(6)]. The ineffectiveness of existed *Leishmania* vaccines is most likely due to the lack of consistent stimulation of helper T cells, a requirement for long-lived protection that usually occurs in response to natural and low profile persistent infections [(7)]. New antigens with such properties to stimulate memory T helper cells, a perquisite for a potent and lasting immunity, are still in need for constructing effective new vaccines [(8)].

Preparation of soluble *Leishmania* antigen (SLA)

Soluble *Leishmania* antigen (SLA) was prepared according to Dumonteil *et al* (2003) with slight modification [11].

Briefly, a number of 1~ $2*10^9$ late log *L. major* M379 (stationary phase) was washed 3 times with sterile 1xPBS (4000 g at 4° C). Parasite pellet was then re-suspended in *Leishmania* Buffer (a 100 mMTris pH 7.3 buffer with 1 mM EDTA, 0.5 mM Phenylmethanesulfonyl fluoride (PMSF) and 2.5 µg/ml Leupeptin - Sigma). The suspension was sonicated for 20 minutes. The sonicated pellet was centrifuged for 30 min at 13,000× rpm. Supernatant was dialysed against 3 litres of cold PBS for overnight with at least two changes of 1xPBS. SLA was sterilized by passing through 0.25 µm filters (Sartorius), and then kept at -20°C for further investigations.

The total protein concentration of extracted protein was measured using Bicinchoninic Acid Protein Assay Kit according to manufacturer's protocol.

Preparation of *Leishmania major* cDNA

The *L. major* gp63 cDNA construct (PNUT), a kind gift by Dr. Selman Ali, Nottingham Trent University, Nottingham, UK, was bulked up by transformation of *Escherichia coli* followed by purification using Quia-gen EndoFree (West Sussex, UK) plasmid purification Maxi Prep Kits. The construct was sequenced by MWG Biotech using 5'-GTCTCCACCGAGGACCTCAC-3', 5'-GTTCAGCGGCCCATTCTT-3', 5'-TCTCCGCCTTCATGGACTAC-3',5'-CGTGTCCTTGGGTGACAAC -3' and 5'-CAGCACACCCTCCTCACTC-3' primers.

Cloning of *L. major* gp63 into VR1012

Leishmania major gp63 was cloned into VR1012 vector (kind gift by Dr. E. Dumonteil, Laboratorio de Parasitología Yucatan, Mexico.), which contained a mammalian antibiotic resistant gene. L. major gp63 was first amplified by PCR using 5'-TGTCGATATCCTATGCGTGGGCTGGA-3' and 5'-TCTGAGATCTGGGGAGGGGTCACAGG-3' forward and revers primers containing restrection site for *EcoRV* and *BgIII* restrection enzymes respectively. VR1012 vector and amplified gene were digested using the same restriction enzymes. Then, *lmajgp63* gene and the digested vector were ligated using a DNA ligase enzyme. The presence of the *lmajgp63* gene in VR1012 vector was first determined by PCR

The major surface glycoprotein of Leishmania major is a zinc metalloproteinase of 63 kDa referred to as leishmanolysin or GP63, which is encoded by a family of seven genes. GP63, a highly conserved protein, is abundantly expressed in promastigotes, and considered the major Ag determinant recognized by the serum samples of patients obtained from different clinical forms of leishmaniasis, moreover GP63 has an intrinsic ability to stimulate protective immunity and is promising vaccine candidate against leishmaniasis [9].

It has been reported that the mode of administration of the DNA vaccine can influence the type of immune response induced by the vaccine. Intramuscular injection of naked DNA was one of the first method described for gene immunization which has been reported to lead the immune response toward Th_1 type while application of gen gun-mediated delivery, gold particles covered with plasmid DNA is very effective at driving plasmid into the cells of the epidermis and requires far less DNA than needle injection [10].

MATERIALS AND METHODS

Animals and parasites

Fifty non pregnant female BALB/c mice (4-5 weeks old) were obtained from animal unit at College of Medicine/ University of Baghdad. Mice were bred in standard mice cages for ten weeks and fed on standard mice ration.

Ten ml vial frozen in liquid nitrogen contain *L. major* M379 strain (College of Science and Technology-Nottingham Trent University/ UK) was used in this study.

Parasite was grown in Drosophila Schneider media (Lonza), supplemented with 10% fetal calf serum (FCS) at 37°C in CO_2 incubator for 24-48 hour. For parasite counting a volume of 10µl from the culture at specific time points was transferred to 1 ml eppendorf tube containing 90µl of 2% Paraformaldehyde. After mixing, 10µl of fixed parasites were transferred to a second eppendorf tube containing 90µl 1xPBS, and counted using Neubauer Hemocytometer.

Sampling procedure

After two weeks from the last vaccination, at least 1 ml blood sample was collected in1.5 micro-centrifuge tube from the heart of each mouse by using insulin syringe. Sera were obtained from blood samples by centrifugation and kept at -20℃ for further investigation.

IMMUNE RESPONSE OF BALB/C MICE AGAINST GENETIC VACCINATION WITH *LEISHMANIA MAJOR* GP63 GENE (*LMAJGP63*)

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ABSTRACT

Leishmania major glycoprotein 63 (*lmajgp63*) gene was used in this study as DNA vaccine candidates. Gene was inserted into VR1012 plasmid by using standard molecular biology protocols, resulting in preparation of *lmajgp63*/VR1012 plasmid. Vaccine either used as naked or gold particles coated DNA vaccine in immunization of females Balb/c mice. Animals were immunized at week 0, 2 weeks and 6 weeks. Dermojet needle free injector had been used to deliver gold particles coated DNA vaccine intradermally (I/D) while ordinary needle injection was used to deliver naked vaccine intramuscularly (I/M). Immune response for each vaccinated group were detected, two weeks after the third administration of the vaccines, by estimation of serum concentration of IL-2, IL-4, IL-10 and INF-γ, as well as anti-soluble *Leismania* antigen (anti-SLA) IgG titer, by ELISA test. The results demonstrated the effectiveness of DNA vaccines in induction immune response comparing to control groups ($P<0.05$). The highest serum concentrations of IL-2, INF-γ and anti-SLA IgG OD value observed in mice group which immunized with gold particles coated vaccine injected I/D (182.10 pg/ml, 1699.20 pg/ml and 0.6101 respectively), while the lowest titer was observed in group vaccinated with naked plasmids injected I/M (103.60 pg/ml, 1183.20 pg/ml and 0.3395 respectively). On the other hand group treated with naked plasmid I/M shows the highest titer of IL-4 and IL-10 concentration (85.30 pg/ml and 53.10 pg/ml respectively), while the lowest titer

amplification using 5'-CTATGCGTGGGCTGGAGC-3' (forward) and 5'-CAGCACACCCTCCTCACTC-3' (reverse) primers. Moreover, to ensure that the sub-cloned gene contains the correct gene sequence and no mismatches had occurred during the cloning procedure the whole gene was subjected to sequence analysis.

Preparation of gold particles coated DNA vaccine

Plasmid construct encoding *L. major* gp63 gene as well as empty vector control vaccine were coated onto 1.0µm gold microcarriers beads using manufacturer's instructions. Briefly, to help the DNA binding to the gold, 200µl of spermidine (Sigma) mixed with 16.6 mg of gold (Bio-rad), the solution was well mixed, followed by addition of 36 µg of construct DNA. After 10 seconds sonication, 200µl of 1mM CaCl2 was added drop wise to the mixture while sonicating. To precipitate the DNA, the mixture was allowed to stand for 10 minutes at room temperature. Precipitated gold-DNA was pelleted by centrifugation at 13,000 rpm for 5 minutes, and the pellet was washed three times with 1ml of 100% anhydrous ethanol (Sigma) with sonication for 30 second each time and spinning for 1 minute at 13.000 rpm, ethanol was removed by pipetting. After last wash gold-DNA pellet was re-suspended in 2ml of 0.025mg/ml Polyvinylpyrrolidone (PVP) in a sterile 15-ml conical tube. The gold PVP solution was then loaded into dried Tefzel tubes using 5 ml syringe. The solution was left to settle for 30 minutes on the Prep Station, so the gold particles settle to the bottom of the tube. The supernatant was gently poured off using the attached syringe. The tube was rotated for 5 second and then left to dry by turning the N2 on, for 5 minutes. The plastic tubing was removed from the Prep Station and re-suspended in PBS. Naked vaccine was prepared without coating with gold particles. DNA vaccines were kept at -20℃ until required.

Immunization with *Imajgp63/VR1012* vaccine

The animals were divided into 5 groups, each group contain 10 mice. Animals were immunized by using Dermojet free-needle injector JI-150 (Akra, France) with 50 µl gold particles coated vaccine , particles coated empty vector or gold particles at week 0, 2 weeks and 6 weeks intradermally, while 100 µl naked vaccine or naked empty vector was injected I/M by using ordinary insulin syringe, immunization protocol is shown in figure 1.