Anti - Glucose Regulated Protein 78 (GRP78) antibody responses in Alum/ALM plus BCG vaccinee, visceral leishmaniasis, and Post Kala- azar Dermal leishmaniasis patients

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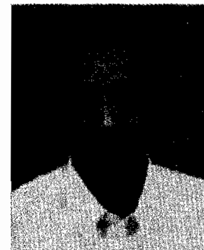

Abstract

Introduction: The Glucose Regulated Protein 78 (GRP78) of Leishmania donovani is considered to be one of the potential Leishmania vaccine candidates.

Patients and methods: Using Enzyme-Linked Immunosorbent Assay (ELISA), we measured IgG antibody responses to GRP78 in 39 healthy Sudanese volunteers vaccinated with Leishmania Alum/ALM + BCG vaccine, 29 patients with visceral leishmaniasis (VL), and 26 patients with post kala-azar dermal leishmaniasis (PKDL).

Results: There was, no significant statistical difference in plasma levels of GRP78 antibodies in immunized and control group (P=0.37). Furthermore, no significant statistical difference in the levels of GRP78 antibodies in the pre and post vaccination plasma samples (P=0.60). Plasma IgG levels to GRP78 was significantly higher in visceral leishmaniasis and PKDL patients compared with control group (P=0.00).

Conclusion: This study concludes that Alum/ALM vaccine does not induce a Th2 type of immune response. It also demonstrated clearly that VL and PKDL are associated with elevation of anti-GRP 78 antibodies and that GRP78 ELISA can be used to confirm diagnosis of Leishmania infections based on the clinical presentation.

Key words: Immunize, cutaneous, Sudan, antileishmanial, antibodies.

Introduction

Leishmaniasis is a major and increasingly public health problem, widely distributed in many parts of the world. The worldwide prevalence of leishmaniases is estimated to be 12 million cases, with an incidence of more than 1.5-2 million new cases per year and approximately 350 million people are at risk of infection and disease[1]. A recent report indicated that about 90% of all cases of visceral leishmaniasis (VL) occur in Brazil, India, Bangladesh and Sudan and that 90% of cutaneous leishmaniasis (CL) cases occur in Brazil, Peru, Iran, Saudi Arabia and Syria[2]. Leishmaniasis is caused by infection with parasite of the genus *leishmania*, and it is not a single disease but a variety of syndromes that are complex[3]. The most severe form is visceral leishmaniasis which is considered an important cause of morbidity and mortality in different parts of Sudan mainly in the eastern region. Case detection and treatment is the only tool of control. Animal reservoir and vector control is impractical and difficult and that the solid immunity observed following infection has suggested that vaccination will be an ideal effective method of control[4]. During visceral leishmaniasis the immune system is skewed towards a Th2 type of immune response with production of IL 10, IL 4 and IL 13 and a large amount of antileishmanial antibodies which are not protective[5]. Recent progress towards vaccine development includes human trials using first generation preparations and animal studies using first and second generation vaccine candidates[6,7]. The recently cloned GRP78 of *L.donovani* has been suggested as a new and a promising *leishmania* vaccine candidate. Recently screening of cDNA libraries using patient serum or mouse serum have identified novel *leishmania* proteins namely glucose regulated protein (GRP78), which have been suggested useful as possible vaccine components[8]. Glucose Regulated Protein (GRP78) is a member of the heat shock proteins (HSP70) family and was initially identified as a protein expressed at an increased level in response to glucose deprivation[9]. The objective of this study was to assess the role of GRP78 in the humoral immune responses (Th2) in healthy individuals vaccinated with Alum-precipitated ALM+BCG vaccine, patients with VL and patients with PKDL.

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Materials and Methods

Study samples: Serum samples of 39 healthy volunteers who were vaccinated with Alum-precipitated Autoclaved *Leishmania major* (ALM) with or without BCG as part of the vaccination program held by the Leishmaniasis Research Group based at the Institute of Endemic Diseases, University of Khartoum, Sudan were studied before and after vaccination. Following informed written consent, serum samples of 29 patients with visceral leishmaniasis and 26 patients with PKDL were collected from the endemic area of VL in Eastern Sudan for the study.

Control sample: Following informed written

consent, 15 females were selected from Ahfad University for Women, with no history of leishmaniasis to be as a negative control. Other negative control samples include 15 individuals who live in an endemic area for visceral leishmaniasis (Koka village), and who are leishmanian skin test [LST] negative and 15 individuals who are suffering from malaria but not *leishmania* were selected from Kosti, an area known endemic for malaria but not for *leishmania.*

Methods

Direct agglutination test (DAT)

DAT was used for the detection of anti-*Leishmania* antibodies in serum samples as described by Elharith et al[10]. The antigen used was a soluble antigen produced from a local *L .donovani* prepared by Dr. Elharith at the Medical School, Ahfad University for Women, Sudan.

Glucose Regulated Protein 78 (GRP78) preparation

The Glucose Regulated Protein 78 used in this study was donated by Dr. Anja Jensen (University of Copenhagen, Denmark), the protein was prepared as follows: Promastigotes were maintained in vitro in RPMI medium supported with 10% fetal bovine serum (FBS) at 26 °c. Using a pool of plasma from Sudanese individuals with visceral leishmaniasis or history of VL, an amastigote cDNA expression library was screened. DNA sequencing was done using overlapping synthetic primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kits (PE Biosystems, Alleroed, Denmark). Sequencing homology analysis was performed using the programme offered in the programme Manual of the Wisconsin Package, Version 10 (1998), Genetic Computer Group, (Madison, WI, USA). Expression of the GRP78 gene was analyzed by Northern Blotting using RNA purified from amastigote and promastigote. Polymerase Chain Reaction (PCR) was done in order to amplify the *L. donovani* GRP78. The polymerase GRP78 DNA fragment of the partial cDNA clone was sub-cloned into E-coli, the protein was purified from the bacterial lysates by affinity chromatography, and the protein concentration was quantitated using the protein DC assay kit from Bio-Red, Germany.

Enzyme- Linked Immunosorbent Assay (ELISA)

ELISA was performed to detect the level of GRP78 antibodies as described by Jensen et al[11], in brief: The wells of Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with antigen (0.1µl /well) by overnight incubation at 4°c. Each well received 100µl. The plates were emptied, and any residual binding capacity was blocked with 150µl of blocking buffer (1% bovine serum albumin, 0.5 M Nacl, 0.1% Triton X-100 in phosphate-buffered saline (PBS), PH 7.2) per well, and then plates were incubated for one hour at room temperature. The plates were washed four times with washing buffer (PBS, 0.5 M Nacl, 0.1% Triton X-100, PH 7.4). Plasma diluted 1:100 in blocking buffer was added (100µl) to each well, and incubation for one hour at room temperature was done. The plates were washed four times, the peroxidase-antibody conjugate (1:6000 in blocking buffer, PH 7.2) was added (100µl) to each well, and incubated for one hour at room temperature. Plates were emptied and washing for four times was done. The color was developed using substrate buffer (0.6% OPD, 0.1M Sodium Citrate, 0.05% H_2O_2) was added (100µl) to each well, and incubated for 5 minutes in dark at room temperature. The reaction was stopped using 20% sulfuric acid; the color density was measured using ELISA reader at 490 nm wavelength. Samples were tested in duplicate and only intra-assay variations of less than 15% between duplicate tests was accepted, if the differences was more than15% the test was repeated. A reference, positive plasma was used in all plates. The cut- off level was determined as the mean ELISA units of the plasma from the non-endemic controls plus three standard deviations.

Statistical analysis

Prior to statistical analysis, data entries were performed using excel and access programs. Student's *t*- test was used in the analysis of data. (Independent *t*- test), P. value less than 0.05 was considered statistically significant.

Results

Direct agglutination test (DAT)

Fourty eight volunteers with normal haematological profiles were screened by the DAT for antilieshmanial antidobies. The reciprocal titers for all volunteers (48/48) were <3200. Sera samples from patients with VL and patients with PKDL showed DAT reciprocal titers more than 3200. Malaria patients who were used as control, two of them (2/15; 13.3%) gave antibody responses above the cut- off level. From the fifteen individuals tested, two (2/15; 13.3%) showed reactivity to the antigen above the cut- off level.

Enzyme linked immunosorbant assay ELISA

Antibody reactivity to the GRP78 of *L. donovani* was measured by ELISA using recombinant protein (rGRP78) and Glutathione S-Transeferase (GST) as a control. Two of the

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volunteers (2/39; 5.1%) who received the Alum / ALM +BCG vaccine showed reactivity to the rGRP78 above the cut- off level before and after vaccination, other vaccinees did not show any change in their reactivity to the antigen. The fifteen female university students who were considered as a healthy control did not show any reactivity to the rGRP78 (0/15; 0%). Out of the 29 visceral leishmaniasis patients tested; 21 patients (21/29; 72.4%) showed antibody responses above the cut- off level in comparison to the healthy control group who showed no reactivity (0/15; 0%) to the antigen. Post kala- azar dermal leishmaniasis patients were tested for reactivity to the rGRP78, eighteen patients (18/26; 69.2%) showed antibody responses to the antigen above the cut- off level in comparison to the healthy control group who showed no reactivity to the antigen **(table 1)**.

Table1: The frequency positive and levels of anti-GRP78 antibodies in *Leishmania* infected and control individuals

Group	Frequency Positive	Mean ELISA Unit
Vaccinees (day 0)**	13.3% (2/39)	22
Vaccinee2 (Day 90)***	13.3% (2/39)	24
Visceral leishmaniasis patients	72.4% (21/29)	96
Post kala- azar dermal leishmaniasis patients	69.2% (18/26)	74
Malaria patients	6.6% (2/15)	23
Leishmanin skin negative individuals	6.6% (2/15)	23
Negative Control Group	0.0% (0/15)	18

** Day (0): time before vaccination,
*** Day (90): number of days after vaccination

Discussion

Drugs for visceral leishmaniasis are expensive and not always available, moreover drug-resistant VL is at increase in Sudan[12]. The fact that there is lasting immunity following convalescence of leishmania infection has suggested vaccination to be the way forward to control the disease[13]. A number of candidate vaccine molecules have been studied in experimental murine[14]. Using screening cDNA libraries, the glucose regulated protein (GRP78) from an amastigote expression library was identified[15]. In this study, we assessed the antibody responses to the rGRP78 in sera collected from patients with visceral leishmaniasis, patients with PKDL, and in healthy volunteers vaccinated with Alum/ALM+BCG vaccine. The data indicated that 72.4% of visceral leishmaniasis, and 70% of PKDL patients gave a high antibody responses to the antigen in comparison to the control group **(table 1)**. The strong immune reaction against GRP78 observed in humans during or following *leishmania* infection could be the result of its large quantity as circulating complexes during *leishmania* infection, the amastigotes are destroyed releasing GRP78 in complex with other proteins that will be taken more efficiently and presented by Antigen Presenting Cell (APC) than soluble antigens[16]. There were no significant differences in plasma level of GRP78 antibodies in volunteers before and after vaccination and the control group, indicating that the vaccine didn't induce a Th2 type of immune response. From the 39 volunteers tested, two individuals showed antibody reactivity to the antigen above the cut-off level before and after vaccination, this might draw attention to the role of $\gamma\delta$ T cells and IFN-γ production. We tested the serum of 15 individuals suffering from malaria (examined by blood film) for their antibody responses to the antigen. Two individuals showed cross-reactivity to the antigen, which could be due to previous exposure to *L.donovani* or *L. major*.

From this study we concluded that using Alum/ALM plus BCG vaccine did not induce a measurable humeral immune response (Th2). However, since serum antileishmanial antibodies are indicators of infection and/or disease and that the rGRP78 was recognized by more than 72% of VL samples and 70% of PKDL, serologically GRP78 may help to complement the standard methods of diagnosis of both VL and PKDL in the Sudan.

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