An Experimental Trial of Vaccination Hamsters Against *Leishmania donovani* Using Attenuated Subcultures

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ABSTRACT

This experiment is a trail to use an attenuated *Leishmania donovani* promatigote as a mean for protection the   hamsters against visceral leishmniasis. By subculturing the promastigotes from 1-18 times in NNN media, it was found that subcultures 16, 17 ,18 were effective in protecting   the hamsters against challenge dose of the parasites.

Key words : visceral leishmaniasis, vaccine, hamster.

17   16   .NNN   18-1

                                                             .   18

INTRODUCTION

Leishmania   species are dimorphic, obligatory intracellular parasites. During their life, these organisms cycle between the midgut of the sandflies in which they proliferate as extracellular promastigotes   and   the   phagolysosomes   of   mammalian   macrophages where they differentiate and grow as intracellular amastigotes (Dey *et al*., 2002).

The genus *Leishmania*   are responsible for a spectrum of human diseases termed leishmaniasis. Visceral leishmaniasis (VL) or kala-azar caused by *Leishmania donovani* (LD) is the most fatal form of leishmaniasis afflicting millions of people worldwide (Bhaumik and Naskar, 2009). World Health Organization (WHO) considers leishmaniasis

 to be one of the most serious, epidemic–prone parasitic diseases of the poor and disadvantaged. An estimated 350 million   people are at risk of *Leishmania* infection worldwide with about 1.5 million new cases of cutaneous leishmaniasis (CL) and 500000 cases of visceral leishmaniasis (VL) reported   each year (Kumari *et al*., 2009). The numerous relapses of *Leishmania* are due to the increasing incidence of drug resistance associated with the toxic anti-leishmanial drug in use (Sundar, 2001). No vaccination is available against leishmaniasis and the fast spreading drug resistance in these parasitic organisms emphasizes the need for a safe, effective vaccine (Bhaumik and Naskar, 2009).

Effective   vaccines for VL   require   their activity to elicit cell mediated immunity (CMI) capable of activating macrophages to a microbicidal   states as the protective immunity in human and experimental leishmaniasis is predominantly of Th1 type (Bhaumik and Naskar, 2009; Awasthi *et al*., 2004). It is desirable to have a suitable animal model for human kala-azar   adequate for the realistic evaluation of vaccine safety and efficiency. Golden hamster (*Mesocricetus auratus*) is the best animal model for human VL in having the same clinic-pathological features, including   a relentless increase in visceral parasitic burdens, progressive cachexia, hepatosplenomegaly, pancytopnea, hyper-gammaglobulinemia and ultimately death (Garg and Dube, 2006; Melbey *et al*., 1998; Al-Harmni, 2009).

Several vaccination strategies using live, killed and defined vaccines have been attempted with particular emphasis on their efficacy against CL instead of VL. The use of live vaccines is very promising since they most closely mimic the natural course of infection and therefore may elicit similarly effective immunity after cure. However its use has been restricted or abandoned entirely due to safety concern (Al-Harmni, 1988; Molan and Al-Harmni, 1989).

Many   studies have established the   efficacy   of inoculating live   attenuated (irradiated) parasites as a vaccination procedure against *Leishmania* in murine models of infection. However, this approach is currently considered not acceptable in human due to the possible persistence of virulent parasites in the inoculums that can potentially cause disease in the situations   of immunodiffiency (Al-Harmni, 1988; Molan and Al-Harmni, 1989; Rivier *et al*., 1999). Other potentially usable live vaccines include *Leishmania* administred at a low dose, drug attenuated *Leishmania* or recombinant *Leishmania* cytokines. However these experimental vaccines have yet to reach the clinical trial stage .Hence no effective vaccine currently exist against any form of human leishmaniasis (Bhaumik and Naskar, 2009; Kumari *et al*., 2009; Al-Harmni, 1988).

MATERIALS AND METHODS

Organisms used:

A strain of *Leishmania donovani* (MHOM / IQ / 1982 / BRC1) provided by Nada Al – Bashir, College of Medicine / Al – Nahreen University, Baghdad. This stock culture was maintained in Biphasic medium NNN (Novy-MacNeal-Nicolle).

Experimental animals :

Eighty seven adult male golden hamsters (*Mesocricetus auratus*) each weighting 45-50 gram were obtained from Syria and maintained under conventional condition.

Cultivation of parasites:

The stock culture of *Leishmania donovani* was maintained successfully in the laboratory through weekly serial subcultures.

Stock cultures were maintained on NNN medium at 26C° in Mc-Contry vials containing 5.0ml of slants solid phase, overlaid with 3.0ml of Lockś solution were inoculated with 0.1ml from stock culture during their stationary phase, subcultures were carried out every 5days.The third subcultures were used to infect experimental animals.

The activity and the virulence of the parasite was maintained by cyclic passage *in vivo* in golden hamsters, the latter were sacrificed their liver and spleen were removed aseptically, and inoculated in a fresh NNN medium and kept at 26C°. Examination of these cultures was performed once a week for a period of one month.

Experiment 1:

Seventy two, 8-10 weeks old male golden hamsters were allocated in to 18 equal groups (4 per group). All the animals were infected intraperitoneally with 100×10^6 promastigotes of *L. donovani* in stationary phase from subcultures (1-18) respectively. Forty days later all animals dissected under aseptic condition to avoid contamination, the liver and spleen were cultivated in NNN medium and incubated at 26 C°, an impression Giemsa stained smears were also prepared from the spleen and liver of each sacrificed animal. Table1.

Experiment 2:

Fifteen male hamsters were divided into three equal groups. The animals of the first group injected intraperitoneally with 100×10^6 promastigotes of *L. donovani* subculture 16. Those of the other groups were injected with the same number of promastigotes from subcultures 17 and 18. Month later, all fifteen hamsters, together with 5 previously uninfected hamsters were challenged with 20×10^6. Forty days after challenge, all animals were dissected under aseptic condition, weights of spleen, liver and spleen length were recorded, the liver and spleen were cultivated in NNN medium, an impression Giemsaś stained smears were prepared from the spleen and liver of each animals. Table 2.

Table 1: The subcultures   effect on the liver and spleen of hamsters   infected Intraperitoneally with 100×10^6 *Leishmania donovani* promastigotes and killed (40) days post infection.

Type of subcultures	Liver cultivation in NNN	Spleen cultivation in NNN	No. of animal
Subculture -1-	+	+	4
Subculture -2-	+	+	4
Subculture -3-	+	+	4
Subculture -4-	+	+	4
Subculture -5-	+	+	4
Subculture -6-	+	+	4
Subculture -7-	+	+	4
Subculture -8-	+	+	4
Subculture -9-	+	+	4
Subculture -10-	+	+	4
Subculture -11-	+	+	4
Subculture -12-	+	+	4
Subculture -13-	+	+	4
Subculture -14-	+	+	4
Subculture -15-	+	+	4
Subculture -16-	–	–	4
Subculture -17-	–	–	4
Subculture -18-	–	–	4

(+)= Viable
(–)=None viable

Table 2: The effect of subcultures on the average spleen, liver weights and spleen length of hamsters infected intraperitoneally with 100×10^6 *Leishmania donovani* promastigotes, challenge with 20×10^6 promastigotes and killed (40) days post infection.

Treatments	No. of animal	Mean liver wt. (gm) ±SD	Mean spleen wt. (gm) ±SD	Mean spleen length (cm) ±SD	Result of inoculation
Control challenge	5	6.33 ± 0.08	0.2 ±0.03	3.4 ± 0.31	+
Subculture 16	5	6.01 ± 0.33♦	0.18 ± 0.01♦	2.7 ± 0.15°	–
Subculture 17	5	5.7 ± 0.20♦	0.17 ± 0.01♦	2.4 ± 0.70♦	–
Subculture 18	5	6.0 ± 0.24♦	0.18 ± 0.08♦	2.4 ±0.71°	–

♦P < 0.05 V control by studentś t-test.
°Not significant V control.
(+)= Viable
(–)=None viable

RESULTS

Experiment 1:

It can be seen from Table 1 that the intraperitoneally inoculated hamsters with 100×0^6 *Leishmania donovani* promastigotes from subcultures 1-15 were positive, this confirmed by the presence of promastigotes in the cultures inoculated with the liver and spleen of these animals in NNN media. In addition to the presence of amastigotes in impression smears prepared from the liver and the spleen. While the animals inoculated with subcultures 16,17 and 18 showed negative results, this result confirmed by the disappearance of promastigotes in the cultures inoculated with the liver and spleen of these animals in NNN media. In addition to the disappresence of amastigotes in impression smears prepared from the liver and the spleen of these animals.

Experiment 2:

Table 2 showed that there was statistically significant variation(Studentś t test $p<0.05$) of mean liver and spleen weight between the challenged control group inoculated with 20×10^6 promastigotes and the animals inoculated intraperitoneally with 100×10^6 *Leishmania donovani* promastigotes from subcultures 16,17 and 18. These observations were supplemented with the disappearance of promastigotes in the cultures inoculated with the liver and spleen of these animals in NNN media. In addition to this disappresence of amastigotes in impression smears prepared from the liver and the spleen.

DISCUSSION

It is to be noted that subcultures (1-15) were infective and the inoculated hamsters have no immunity against a challenge dose ,while the subcultures of 16, 17, 18 were not infective and have the ability to build up an immune response among the inoculated animals. The hamster has long been utilized as an experimental host for *Leishmmania donovani*. The extreme susceptibility of this species is exploited for the detection and propagation of the parasite (Farrell, 1976)

Loss of infectivity and /or virulence of promastigotes of *Leishmania spp*. After prolonged in vitro cultivation is well documented. The promastigotes reduced their pathogenicity when remained for a long period of time and the promastigotes in stationary phase were more pathogenic than the promastigotes in logarithmic phase (Abdul-Rahman and El-Hashimi, 1980).

Several vaccine strains of *L. tropica* have been shown to lose their infectivity for human after 1-5 years in culture, while other strains have maintained their infectivity but show a decrease virulence. Similarly, strains of *L. donovani* have lost their infectivity for hamsters after prolonged cultivation while others have retained infectivity but show a decreased virulence (Nolan and Herman, 1985). The decrease in infectivity seen in prolonged culture may be a reflection of the faster growth of a noninfectious subpopulation that might predominate after prolonged cultivation *in vitro* as has been reported for a strain of *L .tropica* (Handman *et al*.,1983). In a study in which hamsters were infected i.c. with 10^7 promastigotes of prolonged cultured population of *L.donovani* 1S, no parasites could be cultured from the spleen at 16 days postinfection (Giannini *et al*., 1981).

(Nolan and Herman, 1985) concluded that, after   prolonged culture, the parasite is apparently   avirulent, causing none of the classical   disease symptoms   in hamsters (hepato- splenomegay and death of the hamster).

Leishmaniasis are unique among parasitic diseases because a single vaccine can potentially be successful at both   treating   and   preventing   the disease (Bhaumik and Naskar, 2009). Vaccination strategies are based on our current understanding of the characteristics   of an effective anti-*Leishmania* immune response as they have been determined   from   human   and   murine   studies (Vanloubbeck and Jones, 2004). Successful immunization protocol should be able to activate the requisite signaling between   macrophages   and   T cells. IFN γ from Th_1 or CD_8^+ T cells as part of the acquired   immune response, and   from IL-12 activated NK cells as part of the innate   response, mediates macrophage activation, nitric oxide production and parasite killing (Bhaumik and Naskar, 2009).

Resistance to leishmaniasis has been associated with a predominant IFN-gamma production   from the antigen   specific CD_4^+ T lymphocyte population (Th1). These cells are effective in promoting macrophage activation at the site of the lesion, and the intracellular *Leishmania*   are killed in a nitric oxide dependent   manner. In addition, activation of the CD_8^+ T cells have also   been shown to be important in playing   a protective role after *L. major* infection, and for effective vaccination in experimental murine leishmaniasis (Vanloubbeck and Jones, 2004).

(Bhaumik *et al*., 2003) mentioned that during the course of in vitro propagation, *Leishmania donovani*   promastigotes lose their infectivity. The lose of infectivity was accompanied   by modifications of surface   glycoconjugates with concomitant expression of terminal galactose residues. The B1-4 galactosyltransferase enzyme of the *Leishmania donovani* promastigotes found to be developmentally regulated and expressed only in the attenuated parasites, the total protein extracts of the attenuated parasites (LP-CSA late passage complete soluble antigens) could stimulate macrophage respiratory burst activity and could also reduce macrophage parasite burden in vitro experiments (Bhaumik *et al*., 2003).

(Bhaumik and Naskar, 2009) reported that the terminal B(1-4)-galactose –linked glycan induce a novel TLR signaling pathway in macrophage leading to up-regulated IFNγ production with TLR and NF-kB p56 involvement. TLR-4 has been shown to contribute   to the control of parasite growth in both   the innate and acquired immune   response   to *Leishmania* infection, the overall   impact of this   signaling pathway would be the initiation of TH1-type immune response.

(Nylen *et al*., 2001) stated that most   effective protection   against   human leishmaniasis has been   achieved   following   vaccination with live promastigotes. Live parasites had the potential to dramatically induce IFNγ secreting cells often in the absence of proliferation. Dead promastigote tended to have the opposite effects, stimulating proliferation but few cells to IFNγ production. These findings suggest that IFNγ inducing factors are located in the membrane of the parasite and that in the live organisms these factors are modified or accumulated.

(Rivier *et al*., 1999) mentioned that the capacity of gp63 (major surface protein of *Leishmania* promastigotes) to protect mice against *L. major* infection was compared to vaccination using live parasites. In all experiments but one, protection offered by

the purified antigen (without or with adjuvant) did not reach the level obtained using radioattenuated or virulent promatigotes .

Previous attempts at immunization against *L. donovani* in the hamster have yielded primary negative results (Stauber *et al.*, 1958; Al- Qubaisi, 1980) or partial immunity (Farrell, 1976). In these studies, an intracardial or subcutaneous route of inoculation of live amastigotes or promastigotes was used as a method of attempted immunization. It is probable that the direct intravascular or subcutaneous administration of parasites does not induce or induce weak acquired immunity, especially if cell-mediated immunity rather than humoral immunity plays a role in protection against visceral leishmaniasis, in addition a severe challenge may overcome a weak immunity which would be sufficient to enable the test animals to withstand a milder challenging dose.

(Nylen and Gautam, 2010) concluded that vaccination with attenuated paraites may be the solution for preventing leishmanial disease. Several methods have been used to develop live attenuated *Leishmania* parasites including long term *in vitro* cultures, selection for temperature sensitivity, chemical mutagenesis and irradiation. (Kedzierski, 2010) stated that the subunit vaccines tested so far did not lead to development of long term immunity, and the whole cell killed vaccine have been performed disappointingly in the field trail. Thus, the live attenuated vaccine provide an appealing alternative. By mimicking the natural infection, live attenuated parasites can deliver a complete spectrum of antigens to the antigen presenting cells, in principle leading to a better immune response that results in a better protective outcome than observed following immunization with a subunit vaccine.

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