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VACCINATIONS WITH LIVE-ATTENUATED *LEISHMANIA MAJOR* PROMASTIGOTES AND CHALLENGE INFECTION WITH *L. MAJOR* IN BALB/c MICE
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VACCINATIONS WITH LIVE-ATTENUATED *LEISHMANIA MAJOR* PROMASTIGOTES AND CHALLENGE INFECTION WITH *L. MAJOR* IN BALB/c MICE

J. A. ONYALO, D.M. MWALA, C.O. ANJILI, A.S. ORAGO and W.K. TONUI

ABSTRACT

Background: **Currently there is no vaccine available in use against any form of leishmaniases worldwide.**
Objective: **To assess potential of a live-attenuated *Leishmania major* promastigates, for protection against a challenge infection with *L. major* in BALB/c mice.**
Design. **A laboratory based study.**
Setting: **Study was carried out at Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi.**
Results. **The greatest protection against challenge with *L. major* was seen in mice immunised with live parasites (P<0.001) compared to vaccinations with heat killed or soluble antigens. In general, immunised mice produced high level of antileishmanial antibodies and T cell stimulation to their respective antigens.**
Conclusions: **Our live-attenuated parasites produced by serial sub-culture of *L. major* parasites 118 times showed the capacity to induce appropriate cell-mediated immune responses and protection against *L. major* infection in BALB/c mice. Data also suggests that these parasites do not revert to virulence when injected subcutaneously in mice.**

INTRODUCTION

Leishmania is a genus of flagellated protozoan parasites that cause a wide range of human diseases collectively known as leishmaniases. Leishmaniases are worldwide in distribution, accounting for 20 million new cases per year with 1.5 persons at risk (1). The disease is prevalent in many tropical and sub-tropical regions of the world, where it is transmitted via the bite of an infected sand fly. Currently, disease control strategies rely on chemotherapy to alleviate the disease and on vector suppression and personal protection to reduce transmission.

Leishmania major causes cutaneous leishmaniasis in the old World and produces wet skin ulcers, which leave unsightly scars on healing (2). *L. major* infection in Kenya is zoonotic with a wide variety of natural hosts being wild animals, which are little affected by the presence of the parasites. Man is almost always an accidental host when he intrudes into the natural habitat of the wild hosts and vector sand flies (3).The parasite is transmitted by *Phlebotomius duboscqi* (4) and the parasites have been isolated from various rodents such as *Tatera robusta, Xerus rutilus* (5,6), *Mastomys natalensis, Taterillus emini, Aethomys Kaiseri* (7) and a vervet monkey *Cercopithecus aethiops* (8). The disease is endemic in Baringo and West Pokot Districts, Rift Valley Province, Kenya (7,9).

Treatment of leishmaniases generally involves the use of high doses of pentavalent antimonial compounds, sodium stibogluconate (Pentostam) and meglumine antimonate (Glucantine®) (10). However, the cost, duration of administration, toxic nature of antimonial compounds and the tendency for the disease to relapse after an initial successive regime of chemotherapy underscore the need for an effective, safe and a cheap vaccine to be used as an intergrated approach to control leishmaniases.

Previous studies with *L. tropica* and *L. donovani* showed that prolonged *in vitro* cultivation of promastigotes reduced the infectivity and the virulence of these parasites (11,12). The efficacy of such promastigotes (attenuated by prolonged *in vitro* cultivation) to induce protective immunity in the host against the *Leishmania* infections has not been examined. This study sought to evaluate *L. major* promastigotes that have been serially sub-cultured one hundred and eighteen times (118°) for vaccine development in leishmaniasis.

MATERIALS AND METHODS

Mice. BALB/c mice aged six to eight week were obtained and maintained in the KEMRI animal house facility. These experiments complied with all relevant institutional policies. *Leishmania* promastigote cultures and preparation of antigens. Virulent *L. major* parasite (strain IDUB/KE/83=NLB 144) was originally isolated from a female *P. duboscqi*,

collected near Marigat, Baringo District, Rift valley province of Kenya and has since been maintained in BALB/c mice (4). Parasites were aspirated from infected footpad lesions of BALB/c mice and cultured in NNN/Schneider's D*rosophila* insect medium supplemented with 20% foetal bovine serum (FBS), 250U/ml penicillin, 250µg/ml streptomycin and 500g/5ml flourocytocine arobinoside at 25°C. The parasites were grown and harvested at metacyclic promastigote stage. Parasites serially sub-cultured one hundred and eighteen times (118°) were used as live-attenuated parasites. Heat killed antigens were prepared by heat killing of whole promastigotes at 60°C in a water bath for ten minutes. Soluble antigens (SLA) were prepared by subjecting the stationery phase promastigote to several cycles of freeze thawing and finally centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was reconstituted in normal saline. Protein concentrations in antigens were estimated using Lowry technique (13) and then stored at -20°C before use.

Immunisation protocol: Four groups each containing thirty BALB/c mice matched by age and sex were immunised intravenously through the tail vein (14). The first group of mice was immunised with 1 x 10^6 live-attenuated metacyclic parasites in 50µl normal saline. The second group was immunised with 1 x 10^6 heat killed whole parasites in 50µl normal saline. The third group was immunised with 50µg of SLA in 50µl normal saline. The final group immunised with 50µl normal saline formed the controls. All mice were boosted with the same dose of antigen at seven days interval for four weeks.

Determination of humoral immune responses against L. major-derived antigens: Twenty mice from each group were bled one week after the third booster by tail snipping method as previously described (14). Briefly, two haematocrit tubes full of blood were collected from each mouse and blotted onto two opposite spots of a filter paper. Filter papers were dried and stored at 4°C until spots containing serum were cut out and put in an elution tube containing 5% Tween 20 bovine albumin in saline containing phosphate 0.05% Tween 20 (PBST) as a diluent. Enzyme linked immunosorbent assay (ELISA) was used to assay the antibody level in the sera as previously described (14). Rabbit anti-mouse Immunoglobin G (IgG) peroxidase conjugate and 2.2'-azinodi [3-ethyl-benzthiozoline sulfate] (ABTS) peroxidase substrate were used. Optical density was read using an ELISA reader with a 492nm filter.

Lymphocyte proliferation assays: Lymphocytes were obtained from mice spleens as previously described (15). Spleens from immunised animals or control groups were aseptically crushed on a wire mesh using a syringe plunger and mononuclear cells were separate using Ficoll gradients. Cell viability was determined by trypan blue exclusion principle. 3 x 10^6 cells per ml were stimulated *in vitro* (37°C in 5% CO_2) with 50µg SLA per ml or medium alone. After 72 hours, the cultures were pulsed with 0.5µCi [3H] thymidine for 18 hours and cell proliferation evaluated by liquid scintillation counting.

Determination of delayed type hypersensitivity (DTH): Both immunised and control mice were injected in the left hind footpad with 25µg of the SLA (16). This amount of antigen had been determined in preliminary studies to be suitable for DTH elicitation. The induration was expressed as the difference between the thicknesses measured before, 24 or 48 hours after eliciting the injection. The measurements were made using a vernier caliper. Indurations above 0.5mm were recorded as positive (16). Control animals were not tested for DTH responses using the molecule to avoid any sensitisation before challenge infection.

Macrophage activation assay: To determine the parasiticidal efficiency of peritoneal macrophages from immunised mice, these cells were harvested from both immunised mice as previously described (17). Briefly, three weeks after the third booster, peritoneal macrophages from both immunised and naive mice were collected by lavage of the unstimulated peritoneal cavity with RPMI 1640 medium buffered with 25mM HEPES. After three washes in PBS, cells were plated on culture flasks containing buffered RPMI 1640 medium, supplemented with 10% FBS plus penicillin/streptomycin and incubated *in vitro* at 37°C in 5% CO2 95% air. After two hours of incubation, non-adhering cells were removed by rinsing with cold medium while the adhering cells were cultured for fourty hours to allow for adequate spreading. After three rinses and renewal of the RPMI 1640 medium-FBS, the cultured cells were exposed to virulent *L. major* promastigotes and incubated at 37°C in 5% CO2 95% air. After periods of infection at four, twenty four and fourty eight hours, the macrophage monolayers were washed, fixed for five minutes in absolute ethanol and stained for 30 minutes with 10% Giemsa. The level of infection in these cultures was calculated by counting macrophages in random microscopic fields in two separate culture dishes and expressing the number of infected macrophages as a percentage of the total number of macrophages counted.

Infection of mice and determination of parasite burdens in the spleens and liver: One week after the third booster, all the immunised and control animals were infected with 10^6 virulent promastigotes in the footpad. Lesion development was monitored by measuring both infected and uninfected footpads once a week using a vernier caliper and the difference between the thicknesses of the two footpads was reported as the lesion size. At 14 weeks post-infection, all mice were killed and their liver and spleens removed. Pieces of these organs were cultured in NNN overlaid in complete Schneider's medium to determine in the presence of parasites. Impression smears were also made from liver and spleen and Giemsa stained. The number of amastigotes per 1000 organ cell nuclei were counted and the number of parasites (X) in organs was derived from the formula X= LDU/1000 multiplied by the weight of the organ (in mg) and a constant $2x10^5$ (18).

Determination of persistence of the live-attenuated parasites: Two separate groups of mice were infected with either live-attenuated *L. major* or virulent parasites 10^6 stationary phase promastigotes subcutaneously in the footpad. The presence of these parasites was monitored by obtaining aspirates from the infected site six, twelve and twenty four hours post-infection. Some of the material from the aspirates was cultured and examined daily for the presence of promastigotes. Impression slides from aspirates were Giemsa-stained and examined for amastigotes. The same procedure was repeated seven and thirty days post-infection respectively.

RESULTS

Humoral responses against Leishmania-derived antigens: Results showed that mice immunised by intravenous injection with various antigens derived from the metacyclic *L. major* promastigotes produced substantial amount of antibodies as shown in Table 1. However, vaccinations with live-attenuated parasites induced the highest production of antibody titers compared to vaccinations with soluble antigens or heat killed whole parasites ($P<0.05$).

Table 1

Mean antibody responses to L. major soluble antigens following immunisation with the respective antigens

Immunising antigen	Mean optical densities (OD ± S.E)
Live-attenuated parasites	0.283 ± 0.004
Heat killed whole parasites	0.198 ± 0.085
Soluble antigens	0.223 ± 0.040
Controls	0.000 ± 0.000

Cell-mediated responses in immunised mice. Cellular immunity against the various *Leishmania* antigens was assessed in vaccinated animals by delayed type hypersensitivity (DTH) and recall proliferative responses to soluble antigen (SLA) in immunised animals. Results showed that all immunised mice showed DTH responses in recognition to soluble leishmanial antigen 24 hours post challenge (Table 2). However, vaccinations with live-attenuated parasites showed prolonged and sustained DTH responses 48 hours before challenge (Table 2).

Table 2

Delayed-type-hypersensitivity responses in BALB/c mice following a triple immunisation with various leishmanial antigens

Immunising antigen	Mean skin induration in MM ± S.E.	
	24 Hours	48 Hours
Live-attenuated parasites	1.30 ± 0.08	1.00 ± 0.09
Heat killed whole parasites	1.20 ± 0.01	0.00 ± 0.00
Soluble antigens	1.00 ± 0.09	0.00 ± 0.00
Controls	0.00 ± 10.00	0.00 ± 0.00

All the vaccinated mice also exhibited proliferative T-cell responses to SLA, an indication of immunological memory after immunisation. Animals vaccinated with soluble antigen revealed the highest T-cell proliferation (Stimulation index = 4.8) followed by those vaccinated with heat killed whole parasite antigen (Sl = 3.8) whereas the mice immunised with live-attenuated parasites induced the least (Sl = 3.0). However, these responses were not statistically significant (Figure I).

Figure 1

Proliferative response to L. major-derived SLA antigen by mononuclear cells from BALB/c mice following immunisation with various leishmanial antigens

Parasiticidal activities of peritoneal macrophages from immunised mice. The peritoneal macrophages from the various immunisation groups were shown to be capable of eliminating virulent *L. major* promastigotes which were introduced into them, but they differed in their ability to eliminate the parasites depending on the type of antigen that was used in immunising the animals. After 24 hours of infection, only 10% of the resident peritoneal macrophages from mice immunised with live attenuated parasites were still parasitised compared to 13%, 10% and 24% from the heat-killed, soluble antigen and un-immunised mice, respectively. After 48 hours of infection, the number of infected cells reduced drastically with those from mice immunised with live-attenuated parasites promastigotes being 2%, 8% from mice immunised with heat killed promastigotes, 4% from mice immunised with soluble antigens and control group still had 20% of its cells infected (Table 3).

Table 3

Vaccinating antigens	24 hours (%)	Duration in hours post infection 48 hours (%)	72 hours (%)
Live-attenuated	10	2	0
Heat killed whole parasites	13	8	0
Soluble antigens	10	8	0
Controls	24	20	6

Lesion development in BALB/c mice following challenge with virulent L. major promastigotes: Figure 2 shows the mean lesion sizes in mice from each group following challenge with virulent *L. major* parasites and which were monitored for a period of 14 weeks. Results showed that mice immunised with live-attenuated parasites developed significantly smaller lesion sizes compared to those immunised with SLA or heat killed promastigotes ($P<0.001$). Lesion sizes in mice immunised with animals immunised with SLA and heat-killed whole parasite antigen did not vary statistically from those of the controls ($P>0.05$).

Figure 2

Mean lesion size of BALB/c previously vaccinated with various antigens and later challenged with virulent L. major promastigotes

Table 4

Mean weights (grams ±S.E.) of liver and spleen of BALB/c mice at 14 weeks post-infection

Vaccination groups	Infected organ	
	Liver	Spleen
Live-attenuated parasites	1.08 ± 0.046	0.43 ± 0.024
Heat killed whole parasite antigen	1.18 ± 0.079	0.45 ± 0.020
Soluble antigens	1.19 ± 0.090	0.46 ± 0.042
Controls	1.33 ± 0.056	0.48 ± 0.030

Parasitaemia levels in the liver and spleen biopsies. Cultures from liver and spleen from mice immunised with SLA and heat killed parasites were shown to be positive for parasites. Those from mice immunised with live-attenuated parasites were shown to be negative. However, the mean weight and sizes of spleens or liver from mice immunised with various antigens did not differ significantly with those of the controls ($P>0.5$).

Persistence of the live-attenuated parasites at the site of inoculation in BALB/c mice: Live-attenuated parasites inoculated in mice were detected from infection site 48 hours post-infection. This was supported from both culture and thin smear results that were all negative. However, mice infected with virulent *L. major* parasites all had parasites in the infection site.

DISCUSSION

To date there is no vaccine against *Leishmania* in routine use anywhere in the world (19). The only vaccination strategy used so far in humans with proven success against cutaneous *leishmaniasis* has required controlled induction of disease with virulent parasites (20). However, this practice has been abandoned because some individuals did not cure these initial purposeful infections (21,22).

The primary focus in modern vaccine research has been on the development of killed whole or fractionated antigen preparations and more recently on recombinant proteins, attenuated live parasites or DNA vaccines (19). In the present study we investigated the ability of live-attenuated, heat killed whole promastigotes and SLA to protect against a challenge infection with *L. major* in BALB/c mice. Results showed that BALB/c mice immunised with live-attenuated parasites produced significantly higher humoral and cell-mediated immune responses compared to vaccinations with heat killed or SLA ($P<0.05$).

BALB/c mice infected with *L. major* parasites develop a progressive infection involving large ulcerating lesions in the infected footpad, loss of foot due to necrosis, metastastic spread of the parasites to secondary cutaneous sites as well as the viscera, and eventual death of the animals (23). Results showed that mice immunised with live-attenuated parasites developed significantly smaller lesion sizes compared to those immunised with SLA or heat killed promastigotes ($P<0.001$). Lesion sizes in mice immunised with SLA and heat-killed whole parasite antigen did not vary statistically from those of the controls ($P>0.05$). No parasites were also detected in viscera from mice immunised with live-attenuated parasites while those immunised with SLA or heat-killed parasites exhibited visceralisation and eventually died of infections with *L.major.*

Previous studies have shown that BALB/c mice injected with avirulent or with irradiated parasites were also protected from infection (24,25). In mouse, *L. major* parasites lacking the gene encoding the enzyme dihydrofolate reductase-thymidylate synthetase (DHFR-TS) induced protection against infection with either *L. major* or *L. amazonensis* (26, 27). An attenuated line of *L. mexicana* was also used successfully to protect against homologous infection (28, 29).Taken together, these data strongly supported prophylactic vaccination with attenuated organisms as a useful approach to human vaccine development. Recent advances to manipulate the *Leishmania* genome by introducing or eliminating genes have the potential to make live-attenuated vaccines much more feasible.

The use of attenuated organisms is very attractive because they are the closest mimic to the natural course of infection and may therefore lead to similar immune responses. Moreover, because of the small load of

antigen delivered by the transient challenge, the immune responses may be skewed even more towards a ThI-type than in natural infections (30,31). Such immunisation will also deliver many more parasite antigens than the limited number possible with recombinant or sub-unit antigens (25).

Our live-attenuated parasites produced by serial sub-culture of *L. major par*asites 118 times have shown the capacity to induce appropriate cell-mediated immune responses and protection against *L. major* infection in BALB/c mice. Our data also suggests that these parasites do not revert to virulence when injected subcutaneously in mice. Our laboratory is now in the process of analysing their genetic profile for future vaccine development in *leishmaniasis.*

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PARAMETERS OF NUTRITION IN SCHOOL GIRLS IN SOUTHWESTERN NIGERIA
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PARAMETERS OF NUTRITION IN SCHOOL GIRLS IN SOUTHWESTERN NIGERIA

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ABSTRACT

Objectives: **To document trends in parameters of nutrition in south-western Nigerian school girls in comparison with earlier reports and provide baseline data for future comparison.**

Design: **A cross-sectional study.**

Setting: **Fifteen secondary schools from the five local governments in Ibadan, Oyo State in south-western Nigeria.**

Subjects: **One thousand six hundred and seventy five apparently healthy female students aged between nine and twenty three years.**

Results: **One thousand six hundred and seventy three questionnaires were analysed. The mean age, mean height and mean body weight were 15.45 years ± 2.06 (SD), 154.98cm ± 8.4(SD) and 46.09kg ± 8.8 (SD) respectively. The mean body fat was 11.12kg ± 4.6 (SD); mean lean body mass (LBM) was 34.96kg ± 4.6 (SD); mean total body water (TBW) was 25.17L ± 3.3 (SD) and the mean body index (BMI) was 19.07kg/m^2 ±2.7 (SD). Girls from upper socio-economic background had significantly higher values of anthropometric measurements and body composition for each age than lower socio-economic class girls. Compared with their peers from another Nigerian city investigated two decades ago, girls in this study were significantly lighter and shorter.**

Conclusion: **The study revealed a decline in nutritional parameters among adolescents. The implications of these findings for the reproductive health of Nigerian women are discussed. Serial studies to monitor trends in adolescents are recommended.**

INTRODUCTION

Nutritional status is an index of health and well-being at both the individual and population levels. Studies assessing indices of nutrition have been sparse in Nigerian school girls. Fewer studies still have evaluated body composition in Nigerian girls. Available reports however suggest lower values of anthropometric measurements in developing countries compared with the developed world, and consequently a poorer level of nutrition (1).

Nutritional assessments need to be updated frequently in view of the dynamic nature of the interactive factors. Prominent among these factors is the role of the national economy. The economies of many developing nations have been in recession with predictably deleterious effects on their populations; a decrease in body weight and body fat has been shown to be associated with inadequate food and energy supplies on a national level (2). It is against this background that we set out to document some parameters of nutrition in school girls with a view to evaluating the trend in comparison with earlier reports and provide baseline data for future comparison.

MATERIALS AND METHODS

This cross-sectional study involved 1675 apparently healthy secondary school students randomly selected from 15 public secondary schools from the five local governments within Ibadan city. Selection was done by multi-stage sampling. In each local government, three schools were randomly selected. In the selected schools, one arm in each class (among Junior Secondary School classes 1-3 and Senior Secondary School classes 1-3) was then selected and twenty girls were randomly selected to participate in the study.

Permission for the study was obtained from the supervising Ministry of Education. In each school, the school administration gave its consent and in every selected class, the Tutor explained the nature of the study to all students. The instrument was a pre-tested semi-structured questionnaire, which sought to obtain the date of birth, the mother's level of education and the father's occupation. The social class of the students was derived from the combination of the mother's level of education and the father's occupation as described by Olusanya *et al* (3).

The height (cm) was measured with the girls barefoot and in light-clothing using a standardised metre rule. The body weight (kg) with the girls barefoot and in light-clothing