Montanide ISA 720 is more effective than BCG as an adjuvant for Leishmania killed vaccine in BALB/c mice

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Submitted: 13 Aug. 2009; Accepted: 3 Oct. 2009

Abstract
The aim of this study was to evaluate the immunogenicity and efficacy of Montanide ISA 720 against BCG and alum as an adjuvant for Leishmania killed vaccine in BALB/c mice. Groups of mice were immunized with either, Baccile Calmette Guerin (BCG), aluminium hydroxide (alum) or Montanide ISA 720 (MISA) with killed Leishmania major (KLM) promastigotes antigen and later, sacrificed or challenged with virulent promastigotes. Vaccination with alum-KLM and BCG-KLM produced higher IgG responses than vaccination with Montanide ISA-KLM. Significantly higher lymphoproliferative response was obtained for the MISA-KLM mice than in both the alum-KLM and BCG-KLM vaccinated mice. The BCG-KLM group produced the highest interferon gamma (IFN-\(\gamma\)) while the alum-KLM gave the least IFN-\(\gamma\) responses. Among the three adjuvants, BCG vaccination recorded the biggest lesion sizes, an indication of adverse reaction by BCG while the MISA 720 vaccinated mice showed the smallest cutaneous lesions. There was a good correlation between parasite burden and IFN-\(\gamma\) level indicating IFN-\(\gamma\) response as a sensitive parameter of immune response to the vaccine antigen used. The findings suggest MISA-KLM as a safe and effective immunization against cutaneous leishmaniasis but suggests for confirmation of the results in non-human primate model before clinical trials in humans.

Keywords: adjuvants; alum; BCG; Montanide ISA 720; KLM; vaccination; cutaneous leishmaniasis; BALB/c mice; immune responses.

INTRODUCTION

Leishmania major is the causative agent of zoonotic cutaneous leishmaniasis (CL) and affects millions of people in many parts of the world (WHO/CID/Leish/98.9 Add.1). Despite a lot of efforts to control leishmaniasis, it still remains a major health problem. Current control measures rely on chemotherapy to alleviate disease and on vector control to reduce transmission. To date, there is no vaccine against any form of leishmaniasis for general human use (Khamesipour \textit{et al.}, 2006). Protection from CL has been achieved through artificial infection, a technique called ‘leishmanization’ (Guirges, 1971). However, the use of live vaccines has had many problems including the development of large uncontrolled skin lesions, exacerbation of psoriasis and other skin diseases and even immunosuppression (Serebryakov \textit{et al.}, 1972; Modabber, 1975). Leishmania replicates intracellularly in macrophages, and effective control requires macrophage activation and nitric oxide (NO)-mediated killing in response to Th 1-produced cytokine IFN-\(\gamma\) (Stacey \textit{et al.}, 1999). Killed antigens that could be safer as vaccines generally require an adjuvant for induction of a strong Th1 immune response in murine models. Formalin-killed promastigotes have been used in several studies as antigens in vaccine candidates against leishmaniasis (Holbrook \textit{et al.}, 1981; Cook and Holbrook, 1983; Holbrook and Cook, 1983). The availability of hundreds of different adjuvants has prompted a need for identifying rational standards for selection of adjuvant formulations based on safety and sound immunological principles for human vaccines (Alving, 2002). The use of Bacille Calmette-Guerin (BCG) as an adjuvant is regarded as an acceptable practice in man, and at present this adjuvant is routinely used in vaccination and immunotherapy trials against leishmaniasis (Convit \textit{et al.}, 1989; Bahar \textit{et al.}, 1996;
Montanide ISA 720 is more effective than BCG as an adjuvant

Sharifi et al., 1998; Momeni et al., 1999; Khalil et al., 2000).

Bacille Calmette-Guerin has been used successfully for anti-Leishmania immunotherapy in South American patients without side effects. Bacille Calmette Guerin vectors carrying gp63 have also been used successfully to induce protection in the L. major system (Abdelhak et al., 1995; Cabrera et al., 2000). A study done in the year 1999 (Araujo et al., 1999) concluded that, vaccination of BALB/c mice with a combination of BCG and killed Leishmania promastigotes reduces acute Trypanosoma cruzi infection by promoting an IFN-γ response. The number of adjuvants currently approved for use in humans is quite limited and of those adjuvants that have been tested in humans, alum has had the greatest clinical use and is relatively nonreactogenic (Kaslow et al., 1994). Alum is currently the only Food and Drug Administration-approved adjuvant in clinical use in humans (Tonui et al., 2004). Aluminum salts are inexpensive, safe and simple to formulate (Bomford, 1989). In a previous study (Kenney et al., 1999), a single dose vaccine with heat killed Leishmania amazonensis antigens using recombinant human IL-12 and alum as adjuvants was safe and fully effective against L. amazonensis challenge of Rhesus monkeys model of cutaneous leishmaniasis while vaccination with the recombinant human IL-12 alone showed only partial protection. Vaccination with alum-precipitated autoclaved Leishmania major with BCG resulted in complete protection of Leishmania donovani infected Indian langur monkeys (Misra et al., 2001). Montanide ISA 720 has been recommended by the manufacturer for clinical trials in humans (Gomez et al., 1999). This adjuvant has been used in malaria, HIV and cancer vaccine trials (Kenney and Edelman, 2003). A vaccine against HIV-1 comprising MISA 720 combined with TAB9 (a multi-epitope polypeptide comprising the central 15 amino acids of the V3 loop from six HIV-1 isolates) induced seroconversion in all immunized volunteers with production of antibodies that were broadly reactive against the V3 peptides included in the protein (Toledo et al., 2001). Anti TAB9 lymphoproliferative responses were observed and sera from all immunized volunteers were reactive against gp120 in Western blot. In immunotherapy against cancer, MISA 720 combined with T-cell peptide epitopes from gp100, melanoma-associated antigen recognized by T cells (MART-1), tyrosinase, and melanoma-associated antigen-3 (MAGE-3) induced delayed-type hypersensitivity (DTH) skin test responses in vaccinated patients with stage IV melanoma (Hersey et al., 2005). This was indicative of cellular immune response.

Montanide ISA 720 formulated in recombinant malaria subunit vaccine was found to be immunogenic, giving partial protection in Plasmodium vivax infection (Myriam et al., 2005). In another study MISA 720 combined with a candidate transmission-blocking vaccine against Plasmodium vivax, Pvs25 was found to be effective in the maintenance of a lasting transmission-blocking immunity (Collins et al., 2006). Further studies indicated MISA 720 as being very effective in inducing high proliferation of peripheral blood mononuclear cells and in the release of cytokines (IL-2 and IFN-γ) when used in combination with Plasmodium falciparum malaria vaccine candidate, ICC-132. In the reported malaria vaccine study, high levels of specific antibodies were also observed (Oliveira et al., 2005).

In leishmaniasis vaccine studies, although MISA 720 adjuvant combined with recombinantly produced histone-1 antigen was demonstrated to generate a durable cellular response that was sufficient to control infection in the majority of Vervet monkeys immunized with the vaccine (Masina et al., 2003), studies on this promising adjuvant as a potential ingredient in Leishmania vaccines are very limited. The objective of the present study was to evaluate immune responses and efficacy of MISA 720 against alum and BCG as adjuvants for Leishmania killed vaccine in BALB/c mice. To the best of our knowledge these adjuvants have never been tested before in combination with formalin-fixed Leishmania promastigotes. Our results show that both BCG and MISA 720 are useful in inducing cell mediated immune response and both are found to have significant reduction in parasite numbers but BCG was found to have inflammatory responses. Both BCG and MISA 720 are potential adjuvants for Leishmania vaccines but unfortunately BCG is associated with cutaneous lesions. Alum is a weak adjuvant as it could not induce protective Th 1 response. Among the three adjuvants, MISA 720 is found to be most appropriate as an adjuvant for Leishmania vaccine because of its high efficacy and safety levels.

MATERIALS AND METHODS

Leishmania parasites

Leishmania major isolate, NLB-144, originary isolated from Phlebotomus duboscqui in Baringo District, Kenya and maintained in BALB/c mice by serial subcutaneous passage, was used in this study. An aspirate from the foot of an infected BALB/c mouse was cultured in complete M199 medium (Sigma, , St. Louis, Mo) and incubated at 25°C. Stationary phase promastigotes were harvested by centrifugation at 2500 rpm for 15 minutes at room temperature. The resulting pellet was washed three times by centrifugation in sterile Phosphate Buffered Saline (PBS) and then enumerated in haemocytometer before the parasites were used.
Preparation of Soluble Leishmania Antigen (SLA)

Antigen was prepared following the method described (Gichuru et al., 1995). Briefly, promastigotes were washed and sonicated at 18 kHz for five periods of 45 seconds each on ice, separated by intervals of 1 minute. The sonicated material was rapidly frozen and thawed three times in liquid nitrogen for extraction of whole soluble protein. The parasite suspension was centrifuged at 10,000g for 30 minutes at 4°C. The protein concentration of the supernatant was determined using Bio Rad protein assay kit (Bio Rad) and stored at –70°C until use. This antigen was used for coating ELISA plates for antibody assay.

Preparation of Formalin-fixed Leishmania major antigens (KLM)

For vaccine antigens, in vitro lymphocyte proliferation and cytokine secretion assays, L. major promastigotes were harvested at stationary phase and washed three times in sterile PBS as described before. Parasites were fixed in 1% formal saline for 1hr and then washed three times in PBS as above. They were counted in haemocytometer counting chamber and resuspended in a concentration of 5x10^5/ml in sterile PBS and stored at -70°C until required.

Adjuvants

Montanide ISA 720 (Seppic France), Aluminium hydroxide and Bacille Calmette Guerin (Pasteur Institute of Iran) were used as adjuvants along with formalin-fixed Leishmania major promastigotes.

Experimental design and Immunization protocol

Eight to ten week old male BALB/c mice were acquired and maintained in the rodent facility of the Institute of Primate Research (IPR), Nairobi, Kenya, throughout the experimental period. The mice were divided into 5 groups of 12 mice each and treated as follows: Group 1 received alum plus formalin-fixed Leishmania major promastigotes (KLM), group 2 received BCG plus KLM, group 3 was injected with KLM alone, group 4 received MISA 720 plus KLM and group 5 was injected with phosphate buffered saline (PBS) only and served as the negative control group. Two booster vaccinations of the same vaccine components and amounts were given at four and six weeks following the initial vaccination. In all the vaccinated groups the amount of KLM per vaccine dose was 1x10^7. All the vaccines were reconstituted in PBS to make a total of 100µl per dose and delivered intraperitoneally with a syringe and needle. The adjuvant dosages were 1 mg alum, 10µl of BCG, and 50µl of MISA 720. In total, 300µl of vaccine (promastigotes plus adjuvant) were injected to each experimental mouse. Two weeks after the third vaccination, mice from each group were either sacrificed (at least five mice per group) for immunological assays or challenged (six mice per group) with 1x10^6 virulent L. major parasites for vaccine efficacy evaluations. The Institute of Primate Research committee guidelines and procedures for Animal care and Handling were well followed.

Challenge of mice with virulent Leishmania major parasites

Two weeks after the third vaccination, 6 mice from each group were challenged with virulent L. major promastigotes. Stationary phase promastigotes were prepared as described in section 2.1 and counted. The right hind footpad was swabbed with 70% alcohol and allowed to dry as described (Macharia et al., 2004). The footpad was infected by subcutaneously inoculating 50µl of PBS containing 1x10^6 promastigotes. The thickness of the infected footpads was measured weekly for 8 weeks using vernier caliper. Increase in footpad thickness was expressed as the difference between the infected footpad and the same footpad prior to infection as described (Solbach et al., 1986; Tonui et al., 2004).

Microtitration assay for Leishmania major from infected footpad

Culture microtitration for Leishmania has been described before (Titus et al., 1985; Buffet et al., 1995). Briefly, challenged mice were sacrificed and infected footpads removed between the ankle joint and toes and then homogenized with a tissue grinder in 3ml of complete Schneiders Drosophila insect tissue medium. Under sterile conditions, serial dilutions of tissue homogenate were prepared in wells of 48-well culture plates and incubated at 25°C. Thirty-six hours and 6 days of incubation, parasites were counted in haemocytometer counting chamber under an inverted microscope and the mean counts compared. The one in four dilutions was selected as the best dilution for the microtitration assay for data analysis.

Cell proliferation Assay

Spleen cells were harvested aseptically and purified with Ficoll gradients (Sigma, St. Louis, Mo) to obtain splenic lymphocytes (PBL). The cells were adjusted to 3x10^6/ml in complete RPMI-1640 (RPMI-1640 supplemented with 10% fetal bovine serum (Flow Laboratories, Irvin, UK) 2mM L-glutamine and 100µg/L gentamicin (Sigma) medium. One hundred microliters containing 3x10^5 cells/ml PBL in complete RPMI-1640 were distributed to each well of a 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark) followed by the addition of 100µl of
Montanide ISA 720 is more effective than BCG as an adjuvant for formalin-fixed \textit{L. major} antigens (5x10^6 /ml) or concanavalin A (con A; 10µg/ml; Sigma). Control wells received 100µl of complete RPMI 1640 medium. Cultures were set up in duplicates and incubated at 37°C in a humidified atmosphere containing 5% CO2 for 5 days for \textit{Leishmania} antigen cultures and for three days for con A cultures. The cells were pulsed with 0.5µci of [Methyl-3H] thymidine (New England, Nuclear Boston, MA; 1.85 mBg/ml) over the last 18 hours and harvested on fibre glass filters (Titertek, microtitration Equipment, UK). Incorporation of the radionuclide into DNA was determined by liquid scintillation spectrometry. Results were expressed as the stimulation index (SI) obtained by dividing the counts per minute of antigen or Con A by the counts per minute of the control cultures.

### Quantification of interferon-gamma (IFN-\(\gamma\))

Purified splenic lymphocytes were adjusted to 3x10^6 cells/ml in complete RPMI 1640 medium and stimulated \textit{in vitro} with either Con A or \textit{L. major} antigens as described (Olobo \textit{et al.}, 1992). Culture supernatants pooled from triplicate wells after 72 hours of stimulation were used to determine IFN-\(\gamma\) using mouse IFN-\(\gamma\)-ELISA kit (Mabtech, AB, Sweden, Code 3321-1H-6) according to the manufacturers instructions. Briefly, polystyrene micro-ELISA plates (Nunc, Copenhagen, Denmark) were coated overnight with 100µl of monoclonal antibody, AN18 (1µg/ml) to capture IFN-\(\gamma\) from supernatants of samples and recombinant mouse IFN-\(\gamma\) standard. Nonspecific binding sites were blocked with 3 % bovine serum albumin (BSA) in PBS/0.05% Tween 20 buffer (washing buffer) for 1hr at room temperature. The plate was washed 6 times with washing buffer before the addition of 100µl of supernatant samples or mouse IFN-\(\gamma\) standards and incubation for 2 hr at room temperature. One hundred micro liters of monoclonal IFN-\(\gamma\) detecting antibody, R4-6A2-biotin (0.5µg/ml) was added per well and the plate incubated for 1 hour at room temperature. Streptavidin-HRP was added to the wells at a dilution of 1:1000 and the plate incubated for 1 hour at room temperature before the addition of 100µ/well of Tetramethlebenzidine (TMB) microwell peroxidase substrate. Optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories,UK).

### Enzyme-linked immunosorbsent assay (ELISA) for antibody detection

The assay was performed as described (Gicheru \textit{et al.}, 1995). Briefly, polystyrene Micro-ELISA plates (Nunc, Copenhagen, Denmark) were coated overnight with 100µl of \textit{Leishmania major} soluble antigen at a concentration of 10µg/ml, diluted in bicarbonate buffer (pH 9.6). Nonspecific binding sites were blocked with 3 % bovine serum albumin (BSA) in PBS/0.05% Tween 20 buffer (washing buffer) for 1hr at 37°C. The plates were washed 6 times with washing buffer before the addition of 100µl of the serum samples and incubation for 2 hr at 37°C. The plates were washed 6 times as above and 100µl of 1:4000 horse radish-conjugated sheep anti-mouse IgG (Rockland Immunochemicals) was used as detecting antibody. Tetramethlebenzidine microwell peroxidase substrate was added to the wells and the plate incubated protected from light for 20 minutes before the optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories, UK). All sera were tested at a dilution of 1:100 and the optical densities for different groups compared.

### Statistical procedures

GraphPad Prism version 4.00 statistical software (GraphPad Software, San Diego, CA) was used to carry out analysis of variance (ANOVA) and Spearman rank correlation tests. The Tukey-Kramer test was chosen for post ANOVA comparisons of multiple groups. A P value of <0.05 was considered indicative of a statistically significant difference.

### RESULTS

#### Antileishmanial antibodies

Based on enzyme linked immunosorbed assay (ELISA), all vaccinated animals were clearly shown to be seropositive when compared to the unvaccinated controls.
Montanide ISA 720 is more effective than BCG as an adjuvant. The optical densities at 630nm from the experimental groups ranged between 0.20 and 0.508 and were all above the absorbance values of the negative control animals whose OD values were between 0.047 and 0.142. There was a significant difference in the production of *L. major* specific IgG between all the experimental and the negative control groups (F=39.320, *P*<0.001) with antibody responses being comparable but higher in both the BCG and alum as compared to the MISA 720 vaccinated groups. The MISA-KLM and the KLM-alone vaccinated groups induced similar antibody responses (Fig. 1).

**Recall proliferative responses to Con A and antigens**

Concanavalin A (Con A) and *Leishmania* antigen specific proliferation of splenic lymphocytes was compared for both vaccinated and the negative control groups. All the animals responded to Con A with minimal variation. The mean SI from Con A stimulated splenic lymphocytes for the various study groups ranged from 111±14.07 to 127±7.3 and there was no significant difference in response to Con A between the vaccinated and control animals (*P*>0.05) indicating the viability of the cells used. The mean *Leishmania* antigen specific stimulation indices ranged between 26 and 72 among the vaccinated groups (Fig. 2). The ability of splenic lymphocytes to respond *in vitro* to the KLM antigen was significantly higher for the three adjuvant-KLM vaccinated groups than for the KLM vaccinated group (F=82.018, *P*<0.0001). The MISA-KLM vaccinated group showed significantly higher antigen-specific lymphoproliferative responses than both the alum-KLM group (*P*<0.01) and the BCG-KLM vaccinated group (*P*<0.001). Responses in BCG-KLM and alum-KLM were similar (*P*>0.05; Fig. 2).

**Figure 2:** Recall proliferative responses to Con A and antigens: Lymphoproliferative responses of *L. major* antigen and Con A stimulated splenic lymphocytes from BALB/c mice vaccinated with KLM delivered alone or combined with either alum, Bacille Calmette Guérin (BCG), or Montanide ISA 720 once and then boosted two times. Control mice received PBS only. Two weeks after the last vaccination mice were sacrificed and splenic lymphocytes prepared for lymphoproliferative assay. n=6 (Alum-KLM, MISA-KLM and control each) and n=5 (BCG-KLM and KLM each). Statistical significance was determined by ANOVA. *, *p*<0.01; **, *p*<0.001 (adjuvant-KLM groups).

**Figure 3:** Interferon gamma responses to Con A and antigens: IFN-γ responses (ng/ml) in BALB/c mice following vaccination with adjuvants plus KLM. Purified splenic lymphocytes were collected 2 weeks after the third vaccination and adjusted to 3x10^6/ml in complete RPMI-1640 and stimulated with 5x10^6 formalin-fixed *L. major* promastigotes or Con A. Culture supernatants were collected from duplicate wells 72 hr following stimulation and the concentration of IFN-γ in the supernatants determined by ELISA. The data represents mean ± SE. n=6 (Alum-KLM, MISA-KLM and control each) and n=5 (BCG-KLM and KLM each). *, *p*<0.01; **, *p*<0.001; ***, *p*<0.0001 (significant levels between adjuvant-KLM groups).

**Interferon gamma responses to Con A and antigens**

The mean IFN-γ values from Con A stimulated splenic lymphocyte culture supernatants ranged from 139.383±3.858 to 148.234±2.465 ng/ml. Cell culture supernatants collected from Con A *in vitro* stimulation for all the groups showed no difference in their IFN-γ response (*P*>0.05; Fig. 3). Antigen stimulated culture supernatants from the vaccinated groups produced higher levels of IFN-γ that ranged between 7.63 and 84.34 ng/ml as compared to levels between 0.465 and 0.86 ng/ml obtained from the unvaccinated (negative control) group of mice. Consequently, compared with the negative control group, all vaccinated groups of mice produced IFN-γ responses which were significantly higher (*P*<0.001). The adjuvant-KLM vaccinated groups produced significantly higher IFN-γ levels than the KLM group (F=853.14, *P*<0.0001) when cells were stimulated with KLM. There was significant differences in the abilities of the three adjuvant-KLM vaccinations to induce IFN-γ responses (F=573.31; *P*<0.0001; Fig. 3). The BCG-KLM vaccinated mice produced IFN-γ responses that were significantly higher than that produced by the MISA-
KLM vaccinated group \((P<0.001; \text{Fig. 3})\) while the IFN-\(\gamma\) responses induced by the MISA-KLM vaccinated animals was significantly higher than the levels produced by the alum-KLM vaccinated group \((P<0.001)\). Among the three adjuvants the BCG-KLM vaccinated mice produced the highest IFN-\(\gamma\) levels, followed by the MISA-KLM group with the alum-KLM recording the least quantities of the cytokine.

**Lesion measurements in *Leishmania major* infected mice**

Comparative lesion sizes showed that there was delayed lesion development in the MISA-KLM vaccinated group up to the third week post challenge when the sizes of the footpads of mice in this group gradually started to increase (Fig. 4). This group maintained the lowest footpad sizes throughout the experimental period. The negative control group showed immediate response to the challenge infection by developing thicker footpads, which increased by \(0.5\pm0.117\) mm by the end of the first week post infection (Fig. 4). Eight weeks post challenge, the footpad thickness of the negative control mice had increased by an average of \(1.4\pm0.092\) mm, leading to marked swelling of the infected footpad. The BCG-KLM vaccinated group, unexpectedly, developed higher lesion sizes as compared to all the other vaccinated groups up to the seventh week when the lesion sizes in this group gradually reduced to lower levels as compared only to the KLM antigen group among the vaccinated mice The alum-KLM and KLM groups showed intermediate lesion development between the BCG-KLM and MISA-KLM at least up to the seventh week post challenge when the KLM vaccinated group showed slightly elevated footpad swellings as compared to the BCG-KLM group. Spearman rank correlation analysis showed no significant correlation between the mean lesion sizes measured at eight weeks post challenge and the mean IFN-\(\gamma\) of the corresponding vaccinated groups \((r=-0.7.000; P=0.2333)\).

**Parasite burden from footpads of BALB/c mice after challenge**

The mean numbers of parasites in the various vaccinated and control groups counted from 36 hours and 6 days old culture indicated a similar trend in the relative parasite numbers at the two time points and as such data obtained on day six of culture incubation was analyzed for determination of efficacy between the groups. Significantly lower levels of parasite numbers were obtained from the vaccinated groups of mice as compared to the negative control animals \((F=293.11, P<0.0001; \text{Fig. 5})\). Similarly, the parasite loads were found to be significantly lower for all the adjuvant vaccinated groups when compared with the KLM group \((F=179.42, P<0.0001)\). Although the BCG-KLM and MISA 720-KLM vaccinated groups showed no significant differences in their parasite loads \((P>0.05)\), both groups recorded significantly lower parasite numbers when compared to all the other groups \((P<0.001)\). Spearman rank analysis of the mean parasite numbers and IFN-\(\gamma\) in the corresponding experimental and control groups indicated significant correlation between the two parameters \((r=-1.000; P=0.0167; \text{Fig. 6})\). This analysis indicated highest mean level of IFN-\(\gamma\)
in the mice group harboring the lowest mean number of parasites.

Figure 6: Relationship between interferon gamma response and parasite burden: Groups of BALB/c mice were immunized or not immunized with killed *L. major* promastigotes with or without an adjuvant and 8 weeks later, sacrificed (for determination of IFN-γ response) or challenged with 1x10⁶ metacyclic *L. major* promastigotes. Mice were sacrificed 8 weeks following challenge and parasite loads determined through culture microtitration. Analysis of the relationship between interferon gamma response and parasite burden in all experimental mice following vaccination and challenge, produced a significant correlation (Spearman rank correlation analysis) between the two parameters (P=0.0167). The figure indicates that, the higher the IFN-γ response, the lower the parasite burden in the corresponding vaccinated and challenged group.

**DISCUSSION**

The first objective of this study was to evaluate immune responses induced by alum, BCG and MISA 720 as adjuvants co-administered with formalin-fixed *L. major* (KLM) promastigotes in susceptible BALB/c mice. The results of this study clearly indicate that, alum, BCG and MISA 720 do exert adjuvant effects on the immune response to KLM, as measured by the production of serum antibodies, recall proliferative responses of splenic lymphocytes in *vitro* and the production of IFN-γ. The antibody measurement results could not isolate a distinctly stronger immunogen among the three adjuvants as the BCG and alum produced antibody responses that were comparable but higher than the levels induced by the MISA 720 adjuvant. These results differ from earlier findings in which peak antibody responses and persistence of parasite specific antibody following human vaccination with MISA 720 formulated with *Plasmodium falciparum* antigen were comparable to those obtained following immunization with the antigen mixed with alum (Oliveira et al., 2005). However, other studies showed that, aluminium-adjuvanted vaccines resulted in higher and more prolonged antibody responses than did other adjuvants (Eickhoff and Mayers, 2002). Montanide ISA 720 did not increase antibody responses to the KLM antigen, indicating that MISA 720 adjuvant may not be a good stimulator of humoral responses in this vaccination protocol. This observation may also point out that, the choice of the antigen used may determine the immune responses stimulated by the adjuvant. Bacille Calmette Guérin induced the highest levels of antibody responses and this was not unexpected as BCG has been shown to be a strong stimulant of both cellular and humoral responses (Sohrabi et al., 2005). The high IgG responses shown by alum confirmed this adjuvant as an inducer of antibodies in vaccines (Misra et al., 2001).

*In vitro* T-cell responses was demonstrated by recall proliferative responses to *L. major* and also secretions of IFN-γ when splenic lymphocytes stimulated with *L. major* antigens. In the murine model of *L. major* infection, it is now well established that protection depends on a cell-mediated immune response with expansion of a Th1 subset of lymphocytes (Moll et al., 1990; Sypek et al., 1993). In this study, specific parasite recall lymphocyte proliferation was demonstrated in all the experimental groups. Marked Con A stimulation was demonstrated in all the animals and there was no significant difference in response to Con A in both the experimental and control animals indicating that, the cells used were viable and active. However, the adjuvants were able to immunopotentiate the KLM as all the adjuvant-KLM vaccinated groups had higher antigen specific recall proliferative responses when compared to the KLM vaccinated group. Cells taken from mice vaccinated with MISA-KLM proliferated more when stimulated in *vitro* with formalin-fixed *L. major* promastigotes than cells taken from mice vaccinated with either alum-KLM or BCG-KLM. This may indicate that montanide ISA 720 adjuvant is more immunogenic, priming lymphocytes more strongly than both alum and BCG adjuvants.

*Leishmania major* studies in vervet monkeys established that, high levels of IFN-γ are produced in self cured animals (Olobo et al., 1992). The results of the current study clearly showed that, high levels of IFN-γ were obtained in the BCG-KLM and MISA-KLM vaccinated groups of mice with the BCG-KLM group producing significantly higher levels of the cytokine than the MISA-KLM group. This finding may suggest that both BCG and MISA adjuvants are good inducers of Th1 cytokines and as such can be used in vaccines intended to induce protection against intracellular pathogens. The amount of interferon gamma induced in cells obtained from mice vaccinated with the alum adjuvant were significantly lower than those produced by cells obtained from both the BCG and Montanide ISA 720 vaccinated animals and this again confirms that, alum may be a better adjuvant for inductions of humoral responses (Misra et al., 2001) and not cellular responses. However, other factors need to be considered before a conclusion can be generated to support this view as it has so far been established.
that alum as an adjuvant will provoke both humoral and cell mediated immune response (Syypek et al., 1993).

The second objective of this study was to find out whether vaccinations with the three adjuvants combined with KLM antigens would protect susceptible BALB/c mice from cutaneous disease caused by virulent L. major challenge. In the mouse model of leishmaniasis, it has been shown that IFN-γ producing CD4+ Th 1 cells are important in the establishment of protective immunity (Liew et al., 1989). Th 1 cells produced IFN-γ and IL-2 confers protection against the disease (Scott et al., 1988). From the current study, although the BCG-KLM vaccinated group induced the highest levels of IFN-γ than either alum-KLM or MISA-KLM, this did not translate into reduced lesion sizes as there was no significant difference in the lesion sizes between the BCG-KLM vaccinated and the control groups at weeks 2, 4, and 6 post challenges. Throughout the experimental period, the BCG-KLM vaccinated group of mice showed significantly higher lesion sizes than both the alum-KLM and the MISA-KLM vaccinated animals.

The unexpectedly high lesion sizes in the BCG-KLM group might have been caused probably by inflammatory arthritis, an adverse effect of BCG vaccination (Smrkovski et al., 1977) which may exclude the use of BCG in human vaccines. The delayed lesion size development and the significantly reduced parasite load in the MISA-KLM vaccinated mice may be attributed to the significantly higher levels of protective IFN-γ observed in this group as opposed to the alum-KLM vaccinated animals. Previous studies had confirmed that, vaccination of vervet monkeys with recombinant histone antigen mixed with MISA 720 showed significant reduction in lesion development without observation of any local or systemic adverse reactions (Masina et al., 2003) by this adjuvant. The effects of alum on KLM in this study were different from earlier experiment in which vaccination with alum combined with autoclaved L. amazonensis promastigotes and IL-12 of Rhesus monkeys, showed that, the animals were protected from infectious challenge with virulent L. amazonensis (Kenney et al., 1999). In another study, alum precipitated autoclaved L. major promastigotes mixed with BCG completely protected Indian Langur from L. donovani infectious challenge by inducing highly protective IFN-γ and antibody responses (Misra et al., 2001). If protection was to be based on lesion sizes only in this study, then MISA-KLM vaccination would be the most effective and alum-KLM would be ranked the second best with BCG-KLM being the least effective. The use of BCG as an adjuvant would be ruled out due to the large cutaneous lesions observed in the mice vaccinated with the adjuvant. These observations indicate that, the use of lesion sizes as surrogate markers of protection in mice to evaluate vaccine efficacy may not be a reliable parameter.

The goal of any vaccination strategy is to give effective protection against subsequent infection and a desirable vaccine would be one that is effective and at the same time causing no disease or adverse reactions in the vaccinee. There was a good correlation between the parasite burden and IFN-γ levels. The data from this study clearly provide evidence that combination of KLM promastigotes with either MISA 720 or BCG as adjuvants may improve the immunogenicity and efficacy of the crude Leishmania antigen in vaccines against cutaneous leishmaniasis in mice. However, although BCG appears to be a better adjuvant as shown by the least number of parasites in the BCG-KLM vaccinated animals, it may lead to inflammatory reaction (Alimohammadian et al., 2002) as evidenced by the largest cutaneous lesions recorded in the BCG group when compared to all other experimental groups. This finding may explain the non-significant correlation obtained between the IFN-γ and lesion sizes. On the other hand, MISA 720 has been shown to be safe and effective (Masina et al., 2003; Oliveira et al., 2005) as an adjuvant for human vaccines. In this study, BCG and MISA 720 have indicated to be useful in inducing cellular immune responses and both were found to have significant reduction in parasites but BCG was found to have inflammatory responses.

Both BCG and MISA 720 are potential as adjuvants for Leishmania vaccines but unfortunately BCG is associated with cutaneous lesions. Alum is a weak adjuvant as it could not induce protective Th 1 response. It was found to induce mainly antibody responses which are not protective in leishmaniasis. Compared to both alum and BCG, MISA 720 adjuvant was found to be the most appropriate for Leishmania vaccines due to its safety and ability to induce protective Th 1 responses. These findings are important as they point to the conclusion that, better formulation of adjuvant-antigen combinations may soon produce effective vaccines against intracellular pathogens.

Acknowledgement

This study was partly financed by a grant from WHO/TDR. We acknowledge Nicolas Fasel for providing us with MISA 720 adjuvant. We are grateful to the Institute of Primate Research director, Dr. Thomas Kariuki for availing experimental animals.

References

Montanide ISA 720 is more effective than BCG as an adjuvant


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