Experimental Parasitology 121 (2009) 29–37

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Vaccination with a novel recombinant *Leishmania* antigen plus MPL provides partial protection against *L. donovani* challenge in experimental model of visceral leishmaniasis

Suvercha Bhardwaj [a], R.K. Vasishta [b], Sunil K. Arora [a,*]

[a] Department of Immunopathology, Post Graduate Institute of Medical Education and Research, Sector 12, Chandigarh 160012, India
[b] Department of Histopathology, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India

ARTICLE INFO

Article history:
Received 20 June 2008
Received in revised form 11 September 2008
Accepted 29 September 2008
Available online 17 October 2008

Keywords:
Leishmania donovani
Adjuvant
Immunoprophylaxis

ABSTRACT

The acquisition of immunity following subclinical or resolved infection with the intracellular parasite *Leishmania donovani* suggests that vaccination could prevent visceral leishmaniasis. The characteristics and *in vitro* stimulating capability of the recombinant proteins expressed by previously identified clones on the basis of their capacity to stimulate an indigenously established *Leishmania*-specific cell line leading to high level of IFN-γ suggested these to be potential candidates for immunoprophylaxis against leishmaniasis. In this study, we investigated the protective efficacy of purified recombinant proteins from two of the identified cDNA clones along with the adjuvant MPL, in a hamster model of experimental leishmaniasis. We demonstrate here that the immunization of animals with one of the recombinant proteins (rF14) having 97% similarity to C1 clone of *L. chagasi* ribosomal protein gene P0 (rLiP0) along with MPL provided partial protection against the virulent challenge of *L. donovani*. The absence of antigen-specific lymphoproliferative responses in these immunized animals may be responsible for the lack of complete and long-lasting protection.

1. Introduction

Visceral leishmaniasis (VL), caused by *Leishmania donovani* complex, is characterized by fever, hepatosplenomegaly, leukopenia and cell-mediated immune defects that may lead to death if untreated (Carvalho et al., 1989). It has also been increasingly recognized as an opportunistic infection in individuals with HIV infection (Pintado et al., 2001). It is evident from studies in both experimental models of infection and human patients that a cell-mediated immune response is responsible for the control and resolution of leishmaniasis (Mauel and Behin, 1987). The observation that recovery from infection confers immunity to reinfection (Howard, 1986) strongly suggests that control of leishmaniasis by vaccination may be possible.

Several vaccine approaches, viz., whole parasite (killed or irradiated promastigotes) (Holbrook et al., 1981), soluble extracts (Scott et al., 1987), synthetic peptides (Spitzer et al., 1999), crude or pure surface antigens (Mayrink et al., 1985), recombinant antigens (Stager et al., 2000), amastigote antigens (Wilson et al., 1995), plasmid DNA (Basu et al., 2005) and immunostimulating complexes (Tewary et al., 2004) have been tried in experimental models but have met with limited success.

Previously our laboratory reported the identification of three novel genes [A2/1 (Accession No. AY377788), B4/1 (Accession No. AY161269), F2/1 (Accession No. AY180912)] from an expression cDNA library that was screened using immune sera and *Leishmania*-specific cell line (LSCL). These clones expressed *Leishmania* antigens capable of stimulating the proliferation of immune cells *in vitro* leading to release of high amounts of type 1 cytokine Interferon-gamma (IFN-γ) (Pal and Arora, 2003). Preliminary study showed rA2/1 to be partially protective in hamster model when used along with complete Freund's adjuvant (CFA) (Arora et al., 2005).

Purified protein antigens and peptides, the favored subunit vaccine candidates, are poor immunogens. Injection of these substances usually induces immunological tolerance unless administered with an adjuvant (Gupta et al., 1993). The mechanism(s) of monophosphoryl lipid A (MPL) adjuvant, a detoxified form of lipid A derived from the lipopolysaccharide of *Salmonella minnesota* R595 appears to be the activation of macrophages and induction of cytokine synthesis (Ulrich and Myers, 1995), which result in increased immune responsiveness to relatively less immunogenic antigens, e.g. malarial sporozoite antigen (Richards et al., 1998), short synthetic peptides (Friede et al., 1993) and viral proteins (Sasaki et al., 1997).

In this study, we evaluated and compared the immunogenic and protective efficacies of purified recombinant proteins from two of

* Corresponding author. Fax: +91 172 2744401/2745078.
E-mail addresses: skarora_in@yahoo.com, skarorain@gmail.com (S.K. Arora).

0014-4894/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2008.09.019

these identified cDNA clones (A2/1 and F2/1) of *L. donovani* in hamster model of experimental visceral leishmaniasis along with the adjuvant – MPL. We demonstrated that immunization of hamsters with rF14 (after subcloning A2/1 is renamed as A6 and F2/1 as F14) along with MPL was able to provide partial protection against virulent challenge of *L. donovani* by resisting the increase in splenic/liver parasite burden and upregulating the cell-mediated immune responses along with the reduced expression of suppressive cytokine interleukin-10 (IL-10). The failure of immunization to induce any memory cells may be the reason for a short-lived protection.

2. Materials and methods

2.1. Animals and parasite

Syrian golden hamsters (*Mesocricetus auratus*) of either sex (4–6 weeks old), reared in departmental facility, were used for experimental purposes with prior approval of the animal ethics committee of the Post Graduate Institute of Medical Education and Research (Chandigarh, India).

L. donovani, strain Dd-8 (MHOM/IN/80/Dd-8) obtained from Central Drug Research Institute, Lucknow, India, was used for experimental purposes. The *L. donovani* strain was continuously maintained by repeated passage through Syrian hamsters. Promastigotes of *L. donovani* were cultured axenically in a biphasic medium containing 3.7% Brain Heart Infusion (Hi-Media, India), 2% agar (Hi-Media) and 15% defibrinated rabbit blood. The solid phase slants were overlaid with Hank's balanced salt solution (HBSS) containing 1 mM sodium pyruvate (BDH, UK), 2 mM glutamine (Sigma–Aldrich, USA), 100 IU/ml penicillin (Gibco, USA) and 100 μg/ml streptomycin (Glaxo, UK). We used 4- to 5-day old promastigote cultures to prepare soluble *L. donovani* antigen (LPSA) as described (Arora et al., 1991).

2.2. Subcloning of genes in pQE-81L

Two cDNA clones A2/1 (now renamed as A6) and F2/1 (now renamed as F14) previously identified to be expressing recombinant antigens of *L. donovani* capable of inducing proliferation of *Leishmania*-specific cells leading to release of large amounts of cytokine IFN-γ (Arora et al., 2005) were used as source of cDNA inserts in subcloning and expression of large quantities of recombinant proteins. Inserts previously cloned in pBluescript were subcloned into expression vector pQE-81L (Qiagen, USA) by directional cloning (Sambrook and Russel, 2001) using restriction enzymes BamH1 and Kpn1. Plasmid DNAs of A6 and F14 were isolated and digested with restriction enzymes BamH1 and Kpn1. The cDNA inserts of A6 and F14 were 2200 bp and 1500 bp, respectively.

2.3. Expression and purification of recombinant proteins

After screening of the transformants for correct insertion of cDNAs, the recombinant proteins from these cDNAs were expressed in *E. coli* M15 [pREP4] strain (Qiagen, USA) and histidine-tagged recombinant proteins were purified using nickel–nitriloacetic acid (Ni–NTA) metal affinity chromatography (Qiagen, USA) in presence of IPTG. The purified fractions were analyzed by 12% SDS–PAGE. Proteins were visualized by Coomassie blue staining and also probed with anti-polyhistidine antibody after western blotting. The molecular weights of recombinant A6 and F14 were ~44 kDa and ~29 kDa, respectively (Fig. 1A and B). The concentration of lipopolysaccharide in proteins was found to be less than 0.1 ng/ml, much below toxicity level for the cells cultured *in vitro*.

2.4. Western blotting

The purified fraction of the recombinant proteins was confirmed by western blot (Fig. 1C) using anti-polyhistidine antibody (Sigma–Aldrich, USA) as per the standard protocol (Towbin et al., 1979).

2.5. Immunization and parasite challenge

Groups of four hamsters were immunized with three doses at 2-weeks interval intramuscularly, of experimental vaccines containing (A) 50 μg of either recombinant A6 or F14 along with 50 μg of monophosphoryl lipid A (MPL) (Sigma–Aldrich, USA) and dimethyl dioctadecylammonium bromide (DDA) (250 μg/dose; Sigma–Aldrich, USA), (B) rA6 or rF14 mixed with complete Freund's adjuvant (CFA) (first immunization) followed by booster dose with incomplete Freund's adjuvant (IFA, second and third immunizations), (C) PBS alone and (D) naive. All the hamsters were challenged intracardially with 10^7 freshly isolated amastigotes as described by Melby et al. (1998) except the naïve group. The hamsters were euthanized at the end of 10 and 12 weeks post challenge (p.c.) and various parasitological and immunological parameters were studied.

2.6. Delayed type hypersensitivity (DTH) response

The DTH response was determined by injecting each hamster in the right foot pad with 10^8 formalin-inactivated promastigotes. The size of the induration was determined at 48 h by subtracting the thickness of the contralateral foot (injected with PBS alone) from the thickness of the foot injected with inactivated promastigotes (Travi et al., 2002).

2.7. Parasite burden

Liver and spleen parasite loads were determined microscopically in Giemsa stained spleen and liver touch imprints. Results were expressed as Leishman donovan unit (LDU) using the formula (Stauber et al., 1958):

$$\text{LDU} = \text{organ weight (mg)} \times \text{number of amastigotes per 1000 cell nuclei.}$$

2.8. Humoral immune response

The level of *Leishmania*-specific IgG or rAg-specific IgG in the sera of control, immunized and unimmunized animals was determined by ELISA (Melby et al., 2001).

2.9. Splenocyte proliferation assay

Spleen cells from hamsters were suspended in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (Sigma–Aldrich, USA). The concentration of splenocytes was adjusted to 2×10^5 cells/well in a 96-well culture plate in the presence of 100 μl of mitogen [Con A (10 μg/ml), Sigma–Aldrich, USA] or LPSA (25 μg/ml) or recombinant antigens A6 or F14 (10 μg/ml each)]. Cultures were incubated at 37 °C in a CO_2 incubator with 5% CO_2 for 3 days in case of the mitogen and for 5 days in case of the antigen followed by addition of 1 μCi of ^{3}H-thymidine (BARC, India) for 18 h prior to termination of the culture. The cells were harvested on to glass fibre mats (Skatron, Belgium) and counted in a liquid scintillation counter (Beckman, USA). Results were expressed as (Garg et al., 2005):

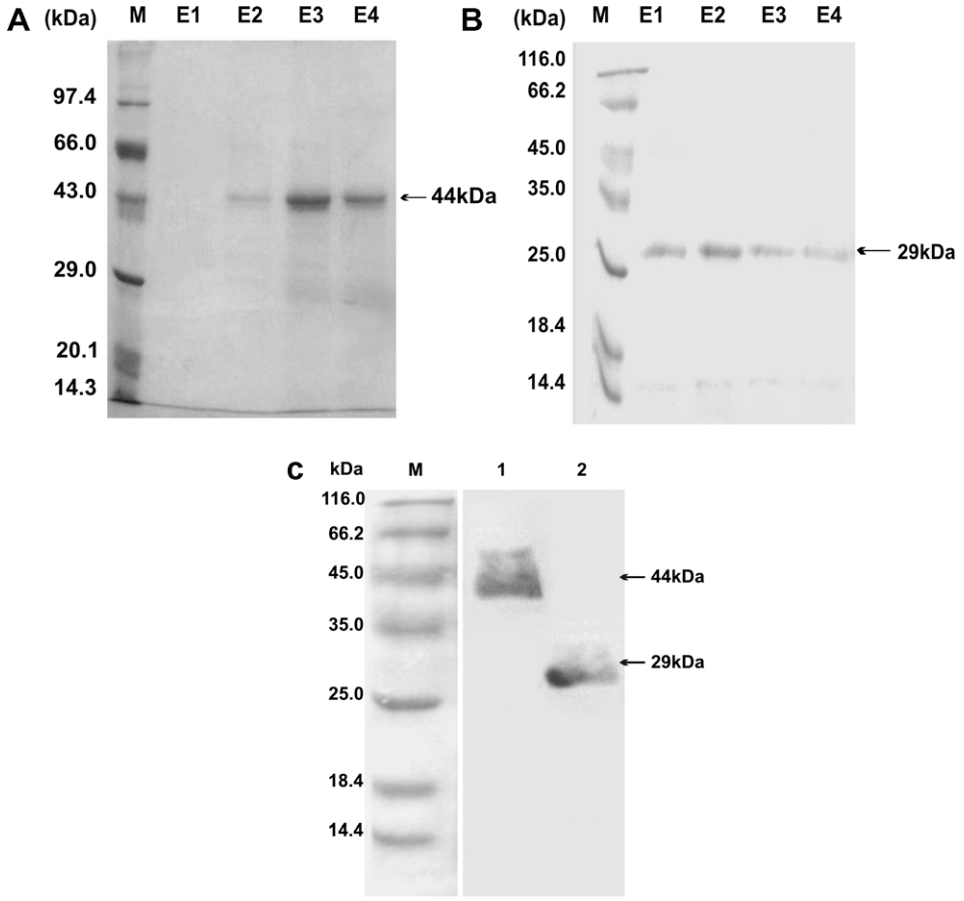

Fig. 1. Purification of recombinant proteins. (A) Coomassie blue stained protein fractions: M, molecular weight marker; E1–E4, A6 eluates (8 M urea, pH 4.5). (B) M, molecular weight marker; E1–E4, F14 eluates (8 M urea, pH 4.5) and (C) probed with anti-polyhistidine antibody after western blotting. The arrow indicates the recombinant A6 and F14 protein bands of 44 kDa and 29 kDa, respectively.

$$\text{Stimulation index (SI)} = \frac{\text{Counts per min in stimulated culture}}{\text{Counts per min in unstimulated culture}}.$$

SI of 20 and above was considered positive in case of mitogen and 2.0 or above in case of antigen.

2.10. Isolation of RNA and oligonucleotides

2.10.1. Reverse transcription-PCR (RT-PCR)

To quantitatively estimate IFN-γ, IL-12p40, IL-10 and IL-4 gene expression in splenic tissues of normal, immunized and unimmunized-infected animals, RT-PCR was done to amplify 245 bp, 234 bp, 213 bp and 238 bp fragments of IFN-γ, IL-12p40, IL-10 and IL-4, respectively. Total RNA was extracted from the spleens (100 mg) with acid–guanidium isothiocyanate–phenol–chloroform (Sambrook and Russel, 2001). Any possible genomic DNA contamination was eliminated by treatment of the RNA with RNase-free DNase. The DNase was then inactivated by addition of 1 μl of 25 mM EDTA and incubation at 65 °C for 10 min. The cDNA, reverse transcribed from the purified RNA (2 μg) in a reaction mixture comprising 20 units of RiboLock™ ribonuclease inhibitor, 0.1 mM of dNTPs, 200 units of RevertAid™ moloney-murine leukemia virus (M-MuLV) reverse-transcriptase and 0.2 μg of random hexamers at 42 °C for 90 min, was used in PCR amplification with 10 pmol of each primer [oligonucleotide primers specific to the hamster HPRT, IL-12, IFN-γ, IL-10 and IL-4 cDNAs from previously published sequences (Travi et al., 2002)] and 1.5 units of Taq DNA polymerase (Roche, Germany). The oligonucleotides were custom synthesized from Sigma–Genosys (India). After an initial denaturation at 95 °C for 5 min, the PCR was carried out for 30 cycles with denaturation at 95 °C for 45 s, annealing at 54 °C for 45 s and extension at 72 °C for 45 s in a thermocycler (Eppendorf, Germany). The last cycle was extended for 8 min at 72 °C. The amplified products were analyzed by agarose gel electrophoresis and documented in a gel documentation system (Image Master VDS, Pharmacia Biotech). The signal intensities of the PCR products were compared to those of hypoxanthine phosphoribosyl transferase (HPRT) gene (391 bp) in the same RNA samples and values were expressed as percent of HPRT gene.

2.11. Superoxide (O_2^-) production

Superoxide production was assayed by the O_2^- dismutase-inhibitable reduction of ferricytochrome *c* (Babior and Cohen, 1981) with some modifications. Briefly, peritoneal exudate cells [5×10^4 cells in 0.1 ml/well 96-well flat bottom tissue culture plates (BD, USA)] from control, immunized and unimmunized hamsters were stimulated *in vitro* with LPS or recombinant antigens A6 or F14 (10 μg/ml each). The supernatant was removed after 48 h and the cells were incubated with 0.2 ml of freshly prepared mixture containing 1 mg/ml of ferricytochrome *c* and 100 ng/ml of phorbol myristate acetate (PMA) in Dulbecco's modified Eagle's medium (DMEM) without phenol red for 90 min at 37 °C. Parallel cultures used as blanks included 30 μg/ml of superoxide dismutase (SOD) in the reaction medium. Supernatants were harvested and OD was measured spectrophotometrically at 550 nm against DMEM. The amount of O_2^- generated in 0.2 ml of reaction mixture was calculated from the difference in OD (ΔE)

at 550 nm in the presence or absence of SOD using the following formula:

$$O_2^- \text{ (nmol)} = 47.7 \times \Delta E \times \text{volume of incubation mixture (ml)}.$$

2.12. Nitrite assay

The presence of nitrite (NO) in culture supernatants of peritoneal exudate cells was assayed by the Griess reaction (Green et al., 1990) in the presence of L-arginine. Peritoneal cells (5×10^4 in 0.1 ml/well) were stimulated *in vitro* with LPS or recombinant antigens A6 or F14 (10 μg/ml each). Supernatants were collected after 48 h. An aliquot of 100 μl of sodium nitrite standards or peritoneal cell culture supernatant samples were seeded in each well (in triplicates) of a 96-well culture plate except for the blank well in which only 100 μl of complete medium (DMEM without phenol red containing 10% FBS) was placed. Subsequently, 100 μl of freshly prepared Griess reagent containing 1:1 v/v mixture of 0.1% *N*-1-napthyl-ethylenediamine (Sigma–Aldrich, USA) in water and 1% of sulphanilamide (Sigma–Aldrich, USA) in 5% phosphoric acid were added to each well and the plate was incubated at room temperature for 10 min. The intensity of the colour developed was read at 550 nm. The nitrite concentration in the macrophage culture supernatant samples was extrapolated from the standard curve plotted with sodium nitrite.

2.13. Histopathological studies

The liver and spleen tissues were fixed in 10% formalin and processed overnight in an auto-technicon. Paraffin blocks of tissues were made and 5 μm thick sections were stained with haematoxylin and eosin (H&E). To determine the degree of cellular response, each infected focus was scored as (1) infected Kupffer cell (KC) with no cellular reaction, (2) fused KCs with few or no associated inflammatory cells, (3) immature granuloma, comprising usually fused infected KCs with limited cellular infiltrate and (4) mature granuloma, showing extensive epitheloid cell development, fused KCs, and organized collar of cellular infiltrate (Murphy et al., 1998).

2.14. Statistical methods

Two-way comparison between the test and control group was performed using Student's *t*-test. Multiple comparisons amongst different groups were performed by analysis of variance (ANOVA). The statistical analyses were made using SPSS software and were considered significant at the level of $p < 0.05$.

3. Results

3.1. Protection parameters

3.1.1. Survival

Under normal course the experimental animals (hamsters) would start showing morbidity due to infection after 10 weeks p.c. with virulent parasites hence the survival of the immunized and unimmunized hamsters at 12 weeks p.c. was considered as the end point of the experiment as well as one of the parameters of protection induced by immunization. At 10 weeks p.c. 100% survival of hamsters was observed in immunized and unimmunized-infected groups. However significant morbidity was observed in immunized as well as unimmunized hamsters at 12 weeks p.c. The hamsters immunized with rF14 + MPL had 100% survival at the end point of the experiment, i.e. at 12 weeks p.c. while most of animals in other groups succumbed to infection between 10th and 12th week p.c.

3.1.2. Spleen weight

A significant increase in spleen weight of infected animals (0.968 ± 0.20 g) was observed from normal spleen weight (0.180 ± 0.02 g) at 10 weeks p.c. ($p < 0.05$). Among the immunized groups, the spleen weight of hamsters immunized with rF14 + MPL was (0.743 ± 0.09 g), 23% less than the unimmunized-infected group (0.968 ± 0.20 g). However, this difference was not statistically significant ($p > 0.05$).

3.1.3. Spleen and liver parasite burden

Spleen and liver parasite loads were determined microscopically in Giemsa stained spleen and liver touch imprints (Fig. 2A). The mean splenic parasite burden in animals immunized with rF14 + MPL was ~46% lower (935 ± 152 LDU) as compared to the unimmunized group (1745 ± 521 LDU) at 10 weeks p.c. which was statistically significant ($p < 0.05$, Fig. 2B). Similar to splenic parasite burden, the liver parasite burden was 36% lower in the group immunized with rF14 + MPL (260 ± 31 LDU) as compared to the unimmunized group (408 ± 130 LDU) at 10 weeks p.c. but this difference was not statistically significant ($p > 0.05$, Fig. 2C).

3.2. Mechanism of protection

3.2.1. DTH response

Induction of delayed-type hypersensitivity reaction based on T-cell responses by rA6 or rF14 was monitored in all the control, immunized and unimmunized hamsters challenged with *L. donovani*. Ten and 12 weeks p.c., DTH response was measured in terms of diameter of erythema developed 48 h after intradermal injection of formalin fixed promastigotes. rF14 administered along with MPL as an adjuvant was able to induce significant DTH response ($27.75 \pm 5.74 \times 10^{-2}$ mm; $p < 0.05$) indicating induction of *L. donovani* specific cell-mediated response (Fig. 3A).

3.2.2. Humoral immune response

The total IgG antibody response against the recombinant antigen or LPSA was examined in the sera of control, immunized and unimmunized animals at 10 and 12 weeks p.c. Vaccination of hamsters with rA6 or rF14 was able to induce significantly high levels of antigen-specific IgG antibodies as compared to the unimmunized-infected group (Fig. 3B). The level of total IgG antibodies against LPSA was however comparable in all the groups.

3.2.3. Splenocyte proliferative response

The splenocytes of control, immunized and unimmunized hamsters were assessed for their proliferative responses against mitogen, LPSA or recombinant antigens. The rF14 and MPL combination was able to induce antigen-specific lymphoproliferative responses (2.4 ± 1.6); however, this value was not statistically significant ($p > 0.05$, Fig. 3C).

3.2.4. Cytokine response after immunization

Basal expression of IFN-γ, IL-12p40 and IL-10 was not detectable in normal hamsters' tissues (Fig. 4A). There was no difference in the level of IL-4 in any of the groups; the level of IL-12p40 was found to be higher in groups immunized with either rA6 or rF14 along with CFA; however, the difference was not statistically significant (Fig. 4B). The expression of IL-10 was lower in the group immunized with rF14 along with CFA and MPL as compared to unimmunized-infected group, but the difference was not significant ($p > 0.05$).

The difference in the expression of IFN-γ between rA6 + MPL group and the unimmunized-infected group was statistically significant ($p < 0.05$, Fig. 4B). The difference in the expression of IFN-γ in other groups and unimmunized-infected group was not statistically significant ($p > 0.05$).

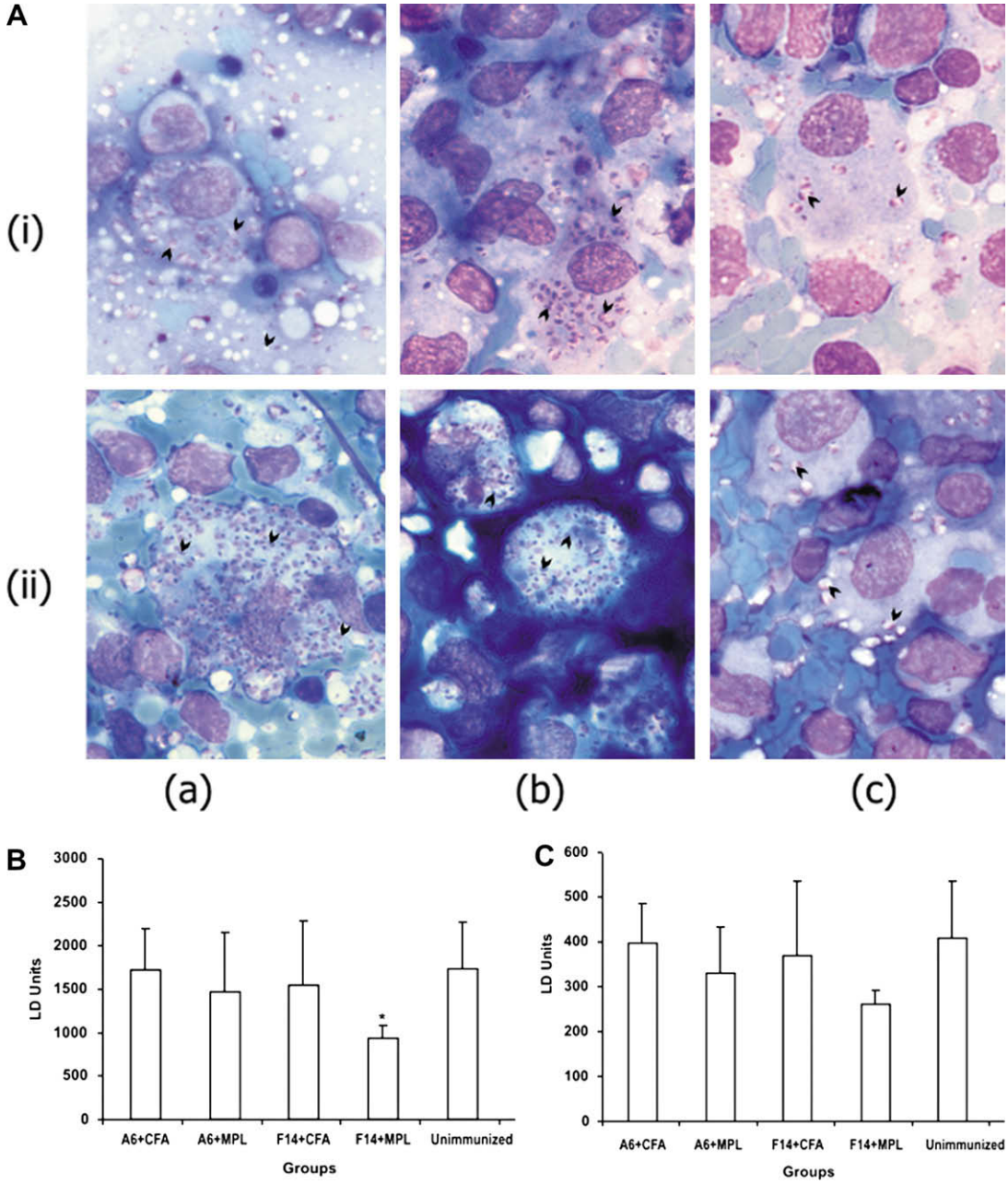

Fig. 2. Evaluation of protection against infection with *L. donovani* in immunized hamsters. Geimsa-stained imprints at 10 weeks p.c. (A). Photomicrographs of (i) liver and (ii) splenic imprints from (a) an unimmunized hamster, (b) an immunized but unprotected hamster (rA6 + CFA) and (c) a partially protected hamster (rF14 + MPL). Intracellular amastigotes are indicated by arrowheads (100× magnification). Splenic parasite burden (B) and liver parasite burden (C) in terms of LDU at 10 weeks p.c. Results are expressed as the mean ± SD of four hamsters per group. Significant difference determined by Student's *t*-test, $^{*}p < 0.05$ compared to unimmunized-infected group.

3.2.5. Generation of the superoxide (O_2^-) anion

The generation of O_2^- was significantly upregulated ($p < 0.05$) in PEC from rF14 + MPL on stimulation with rF14 *ex vivo* (2.79 ± 1.31 nmol) as compared to the unimmunized-infected group (−0.51 ± 1.47 nmol) (Fig. 5A).

3.2.6. Generation of nitrite

NO production was highest in rF14 + MPL group (20.75 ± 2.24) on stimulation with rF14 *ex vivo* as compared to the unimmunized-infected group (7.43 ± 4.19) which was statistically significant ($p < 0.05$, Fig. 5B).

3.2.7. Histopathological responses

Granuloma formation is a key process for the control of *L. donovani* infection and precedes parasite clearance (McElrath et al., 1988). Since *L. donovani* preferentially targets resident visceral macrophages in the spleen, liver and bone marrow, hence the histopathological changes were studied in the spleen and liver. Fig. 6 depicts the various stages of granuloma formation. On the whole the cellular response in liver was restricted to a scoring of 3, i.e. immature granulomas, comprising of fused infected KCs with limited cellular infiltrate in groups immunized with rA6 at 10 weeks p.c. (Table 1). A single animal in rA6 + MPL group demonstrated the formation of mature granuloma in the liver, along with extensive epitheloid cell development, fused KCs and organized cellular infiltrate, i.e. a score of 4. In the groups immunized with rF14 + MPL, 50% of the animals had the score of 4 as compared to the unimmunized ones in the liver. In spleen, all the animals immunized with rF14 + MPL showed the formation of mature granulomas (Table 1).

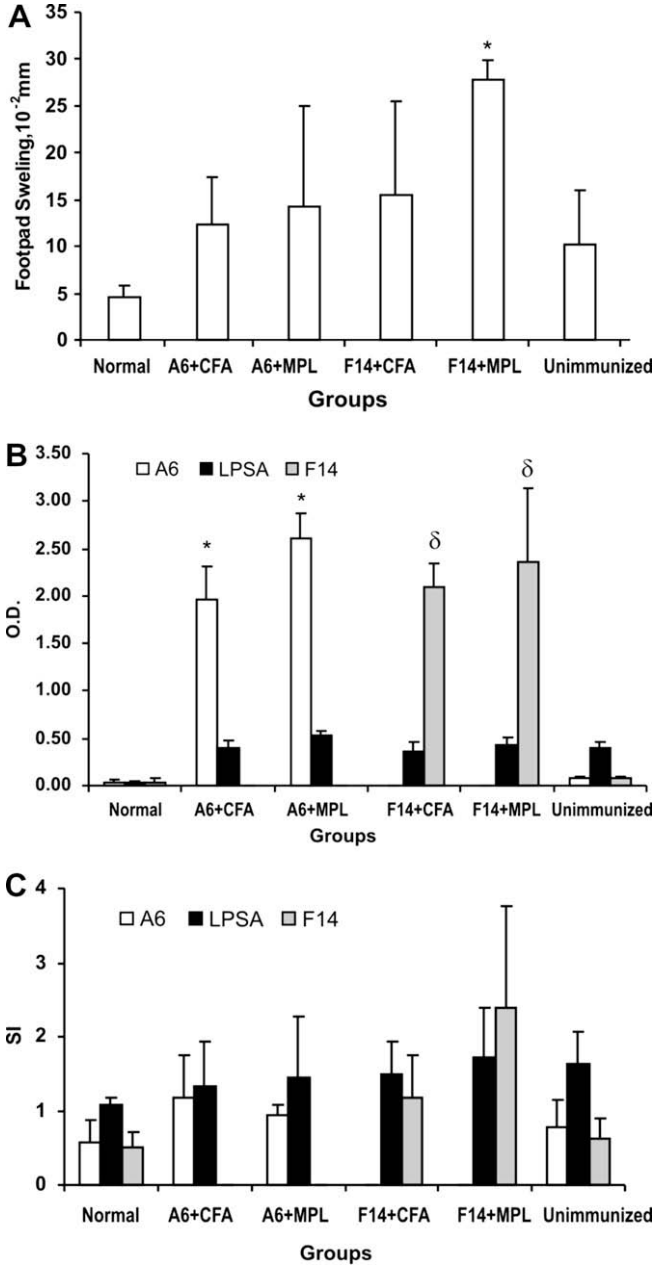

Fig. 3. Mechanism of protection. (A) DTH response to formalin-inactivated *L. donovani* promastigotes in normal, immunized and unimmunized hamsters at 10 weeks p.c. Results are expressed as the mean ± SD of four hamsters per group. *Difference statistically significant as determined by ANOVA ($p < 0.05$). (B) Antigen (rA6, rF14 or LPSA) specific antibodies in the sera of normal, immunized and unimmunized hamsters at 10 weeks p.c. The ELISA results are expressed as the mean ± SD of four hamsters per group. Significance level determined by Student's *t*-test, *$p < 0.05$ compared to unimmunized-infected group, $^{\delta}p < 0.05$ compared to unimmunized-infected group. (C) Lymphocyte proliferative responses in response to rA6, LPSA or rF14 in spleen cells of normal, immunized and unimmunized hamsters at 10 weeks p.c. Results are expressed as the mean ± SD of four hamsters per group.

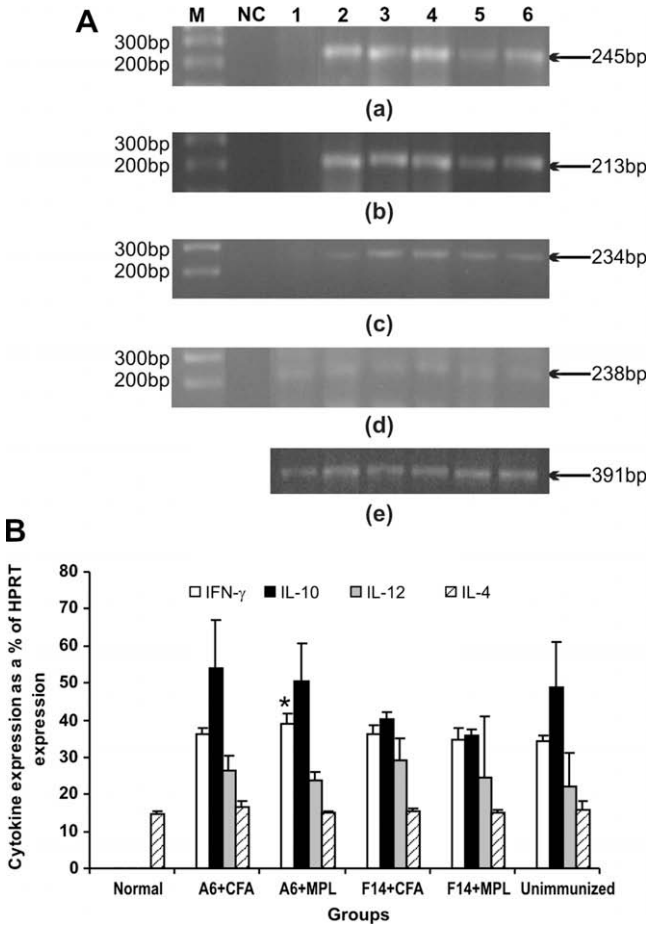

Fig. 4. Ethidium bromide stained 2.5% agarose gels showing amplified RT-PCR products of (a) IFN-γ gene (245 bp), (b) IL-10 gene (213 bp), (c) IL-12p40 gene (234 bp), (d) IL-4 gene (238 bp) and (e) HPRT gene (391 bp), respectively, from spleen tissue of normal, immunized and unimmunized groups at 10 weeks p.c. (A) M, 100 bp ladder; NC, negative control; 1, unimmunized-uninfected; 2, A6 + CFA; 3, A6 + MPL; 4, F14 + CFA; 5, F14 + MPL; 6, unimmunized-infected. (B) Results are expressed as the mean ± SD of the cytokine transcript of four hamsters per group. Significance level determined by Student's *t*-test, *$p < 0.05$ compared to unimmunized-infected group.

4. Discussion

Previous studies from this laboratory had identified three clones on the basis of their capacity to stimulate an indigenously established LSCL leading to release of high level of IFN-γ *in vitro*. The characteristics and *in vitro* stimulating capability of the recombinant proteins expressed by these three clones suggested these to be potential candidates for immunoprophylaxis against leishmaniasis; however, their protective efficacy needs to be confirmed in the animal model. In this study, we have made an attempt to evaluate the protective efficacy of purified recombinant proteins from two of the identified cDNA clones (rA6 and rF14) along with the adjuvant MPL, in a hamster model of experimental visceral leishmaniasis.

The rF14 along with MPL forms the best combination of antigen and adjuvant amongst the tried combinations in our study as all of the rF14 + MPL immunized hamsters survived the entire period of study, i.e. until the termination of the experiment at 12 weeks p.c., whereas most of the animals in other groups succumbed to the lethal *L. donovani* challenge at this time. The splenic parasite burden was also significantly lower in these hamsters at 10 weeks p.c. indicating the efficacy of antigen with MPL combination in providing some level of protection against virulent leishmania challenge. Reed et al. (2003) had also previously demonstrated the efficacy of an antigen formulation Leish-111f when combined with MPL-SE to be protective in terms of reduced lesion size in BALB/c mice against cutaneous leishmaniasis at 14 weeks. A significant elevation of DTH response in hamsters immunized with rF14 plus MPL in comparison to other combinations of rAg and adjuvant as well as unimmunized-infected groups demonstrates the activation of the cell-mediated responses that might be important for provid-

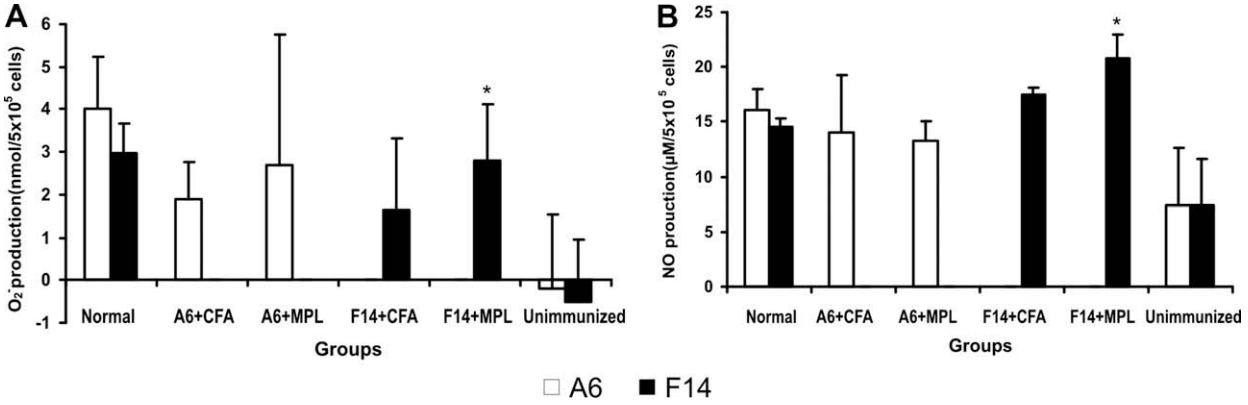

Fig. 5. (A) Superoxide anion (O_2^-) generation in PEC of normal, immunized and unimmunized hamsters at 10 weeks p.c. Results are expressed as the mean ± SD of four hamsters per group. Significance level determined by Student's *t*-test, $^*p < 0.05$ compared to unimmunized-infected group. Nitric oxide generation in PEC of normal, immunized and unimmunized hamsters at 10 weeks p.c. (B) The level of NO in the culture supernatant was evaluated by Greiss method. Results are expressed as the mean ± SD of four hamsters per group. Significant difference determined by Student's *t*-test, $^*p < 0.05$ compared to unimmunized-infected group, $^\delta p < 0.05$ compared to rF14 + CFA and unimmunized-infected group.

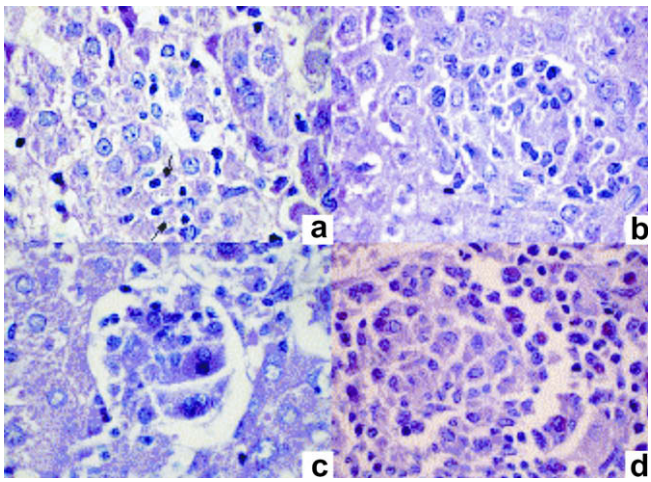

Fig. 6. Scoring of granuloma. (a) Score 1: initial parasitization of resident macrophages (Kupffer cells (KC)) (arrow). (b) Score 2: some KC fusion with few or no associated inflammatory cells. (c) Score 3: immature granuloma with limited cellular infiltrate. (d) Score 4: mature granuloma (H&E stained, 100× magnification).

Table 1
Granuloma formation in liver and spleen of immunized and unimmunized animals at 10 weeks p.c.

Groups		Scoring of granuloma formation	
		Liver	Spleen
A6 + CFA	1	3	3
	2	3	3
	3	3	3
	4	2	2
A6 + MPL	1	3	3
	2	3	3
	3	3	3
	4	4	3
F14 + CFA	1	2	3
	2	3	3
	3	3	4
	4	3	1
F14 + MPL	1	3	4
	2	3	4
	3	4	4
	4	4	4
Unimmunized infected	1	3	3
	2	3	3
	3	3	3
	4	2	3

ing resistance to infection in these animals as a result of immunization.

Apart from diminished cellular responses, visceral leishmaniasis is also associated with elevated levels of γ-globulins as a result of polyclonal B-cell activation (Ghose et al., 1980). This antibody response hardly provides any protection but has been exploited for the diagnosis of *L. donovani* and *L. chagasi* infections (Burns et al., 1993). However, the generation of antigen-specific antibodies along with CMI is considered to be protective (Afrin and Ali, 1997). In our study, hamsters immunized with rF14 + MPL developed significantly higher levels of antigen-specific antibodies as compared to the unimmunized-infected group. These observations are, however, at variance with the studies which report that protection against leishmaniasis is induced by a strong DTH response and almost undetectable amount of antibodies (Bretcher et al., 1992).

It is reported that cytokine-activated macrophages kill ingested *L. donovani* amastigotes by secreting reactive oxygen intermediates (ROI) and/or reactive nitrogen intermediates (RNI), which might act in concert against the parasite (Shiloh et al., 1999) as inhibition of either RNI or ROI pathway prevented macrophage-mediated killing of intracellular parasites (Murray, 1990; Roach et al., 1991). These lethal molecules were also found to be upregulated in the PEC of hamsters immunized with rF14 + MPL combination showing partial protection from a virulent challenge with amastigotes in our study corroborating the report of Yao et al. (1994) that MPL induces the generation of O_2^- and NO in cells *in vitro*.

Fully established kala-azar represents the clinical expression of a net suppressor response, for example, an imbalanced (rather than overtly polarized) Th2 > Th1 cell state (Murray, 2001). Melby et al. (1998) also argued against the paradigm that progressive, uncontrolled leishmanial infection is determined by the induction of a strong Th2 cytokine response and the absence of a Th1 response.

IFN-γ, a Th1 cytokine, plays a key role in the control of infection with many intracellular pathogens, including *Leishmania* spp., and is the cytokine primarily responsible for macrophage activation and killing of the intracellular parasite (Squires et al., 1989). IFN-γ mRNA expression was found to be significantly elevated in rA6 vaccinated hamsters; however, it alone does not seem to be sufficient in controlling the parasite replication. Another cytokine IL-10

has been documented as a potential inhibitor of cytokine synthesis in macrophages (Bhattacharya et al., 2001). We observed down-regulation of IL-10 mRNA expression in hamsters immunized with rF14 + MPL combination. These animals also demonstrated protection at 10 weeks p.c. along with comparable IFN-γ expression which commensurate with the report that after treatment, the level of both IFN-γ and IL-10 is restored to control level (Ansari et al., 2006). In the study of Marques-da-Silva et al. (2005), a significant production of IFN-γ in response to *L. chagasi* antigen and rLACK protein was not associated with a reduction in parasite load either in liver or spleen of vaccinated animals. The plausible explanation for the lack of protection was attributed to significant production of IL-10 induced by the vaccine. More recent studies have also indicated that the vaccines which trigger high levels of IFN-γ do not have protective potential in the presence of high levels of the regulatory cytokine IL-10 (Stober et al., 2005). Moreover, IL-4 and IL-10 act in synergy in the presence of exacerbatory antigens (Roberts et al., 2005). IL-10 thus emerges to be the most appropriate cytokine to serve as an indicator of failure or success of vaccination (Kedzierski et al., 2006).

In infections associated with granulomas such as mycobacteria, selected fungi, bacteria and certain protozoa such as *L. donovani*, these structures are presumed to represent the tissue expression of the successful T-cell-dependent immune response (Mielke et al., 1997; Murray, 1999). In human VL, for example, the presence of granulomas in the liver appears to correlate with spontaneous control and maintenance of infection in a subclinical state (Murray, 1999). Histologically, all the animals in the group rF14 + MPL demonstrated mature granulomas in spleen which might be responsible for the significant lowering of parasite burden in spleen.

The recombinant antigen F14 has 97% similarity to C1 clone of *L. chagasi* ribosomal protein gene P0 (LiP0). Gene expressing rLiP0 antigen used as DNA vaccine partially protected BALB/c mice against *L. major* infection (Iborra et al., 2003). In addition, the C-terminal region of the rLiPO is present in a multicomponent protein that when administered to dogs, using live BCG as adjuvant, conferred protection against *L. infantum* infection (Molano et al., 2003). MPL was able to increase the immunogenicity of rF14 which is evident by the survival of experimental animals till the end point (12 weeks p.c.) of the experiment along with partial protection against the virulent challenge.

Lower parasite burden in spleen and liver accompanied with longer survival of animals challenged with virulent dose of parasite indicates the protective potential of rF14 as a prophylactic antigen. However, the combination of this antigen with MPL could not achieve complete protection and the immunity was short lived. This could be because of non-generation of memory T-cells after immunization with this combination, as distinct subpopulations of $CD4^+$ memory T-cells have been reported to play different roles in providing effective and long-lasting protection against *Leishmania* infection (Zaph et al., 2004), with emphasis on requirements for maintenance of these populations in mice to achieve long-lasting immunity. The absence of Ag-specific lymphoproliferative responses in animals immunized with rF14 + MPL in our study reflects the suboptimal efficacy of this combination to generate memory cells. Another reason could be the nature of animal model and also the route of challenge. Natural infection is characterized by a low-dose infection with 100–1000 metacyclic promastigotes of *Leishmania* and intradermal inoculation (De Almeida et al., 2003). In the present study a high dose (10^7) of freshly isolated amastigotes used as direct i.v. inoculum might have caused an initial assault leading to early homing of parasite in the visceral organs. There is a strong possibility that both of these factors could overwhelm a protective response. It may be worthwhile to further probe the protective efficacy of rF14 along with MPL as adjuvant in the experimental model of VL mimicking a natural intradermal/subcutaneous infection challenge mode.

In brief, rF14 formulated with MPL emerges to be a potential vaccine candidate, as the vaccination conferred partial protection against the lethal challenge of *L. donovani* in hamster model of experimental VL. The failure of immunization to induce any memory cells may be the reason for a very short-lived protection. Further search for a better adjuvant capable of a sustained release of antigen leading to generation of memory cells would be helpful in providing long-lasting protection. However, study demonstrates the potential of rF14 antigen as a novel vaccine candidate against experimental VL.

Acknowledgment

This work is supported by a Grant (BT/PR5232/Med/14/612/2004) from Department of Biotechnology (Ministry of Science and Technology, Govt. of India).

References

Afrin, F., Ali, N., 1997. Adjuvanticity and protective immunity elicited by *Leishmania donovani* antigens encapsulated in positively charged liposomes. Infection and Immunity 65, 2371–2377.

Ansari, N.A., Saluja, S., Salotra, P., 2006. Elevated levels of interferon-γ, interleukin-10, and interleukin-6 during active disease in Indian kala azar. Clinical Immunology 119, 339–345.

Arora, S.K., Pal, N.S., Mujtaba, S., 2005. *Leishmania donovani*: identification of novel vaccine candidates using human reactive sera and cell lines. Experimental Parasitology 109, 163–170.

Arora, S.K., Sinha, R., Sehgal, S., 1991. Use of in vitro method to assess different brands of anti-leishmanial drugs. Medical Microbiology and Immunology 180, 21–27.

Babior, B.M., Cohen, H.J., 1981. Measurement of neutrophil function: phagocytosis, degranulation, the respiratory burst and bacterial killing. In: Clinc, M.J. (Ed.), Methods in Hematology, Leukocyte Function, vol. 3. Churchill Livingstone Inc., New York, pp. 17–18.

Basu, R., Bhaumik, S., Basu, J.M., Naskar, K., De, T., Roy, S., 2005. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. Journal of Immunology 174, 7160–7171.

Bhattacharya, S., Ghosh, S., Jhonson, P.L., Bhattacharya, S.K., Majumdar, S., 2001. Immunomodulatory role of interleukin-10 in visceral leishmaniasis: defective activation of protein kinase C-mediated signal transduction events. Infection and Immunity 69, 1499–1507.

Bretcher, P.A., Wei, G., Menon, J.N., Bielefeldt-Ohmann, H., 1992. Establishment of stable, cell-mediated immunity that makes 'susceptible' mice resistant to *Leishmania major*. Science 257, 539–542.

Burns Jr., J.M., Shreffler, W.G., Benson, D.R., Ghalib, H.W., Badaro, R., Reed, S.G., 1993. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. Proceedings of the National Academy of Sciences, USA 90, 775–779.

Carvalho, E.M., Bacellar, O., Barral, A., Badaro, R., Johnson Jr., W.D., 1989. Antigen specific immunosuppression in visceral leishmaniasis is cell-mediated. Journal of Clinical Investigation 83, 860–864.

de Almeida, M.C., Vilhena, V., Barral, A., Barral-Netto, M., 2003. Leishmanial infection: analysis of its first step. A review. Memorias do Instituto Oswaldo Cruz 98, 861–870.

Friede, M., Muller, S., Briand, J.P., Van Regenmortel, M.H., Schuber, F., 1993. Induction of immune response against a short synthetic peptide antigen coupled to small neutral liposomes containing monophosphoryl lipid A. Molecular Immunology 30, 539–547.

Garg, R., Gupta, S.K., Tripathi, P., Naik, S., Sundar, S., Dube, A., 2005. Immunostimulatory cellular responses of cured *Leishmania*-infected patients and hamsters against the integral membrane proteins and non-membranous soluble proteins of a recent clinical isolate of *Leishmania donovani*. Clinical and Experimental Immunology 140, 149–156.

Ghose, A.C., Haldar, J.P., Pal, S.C., Mishra, B.P., Mishra, K.K., 1980. Serological investigations on Indian kala-azar. Clinical and Experimental Immunology 40, 318–326.

Green, S.J., Crawford, R.M., Hockmeyer, J.T., Meltzer, M.S., Nacy, C.A., 1990. *Leishmania major* amastigotes initiate the L-arginine dependent killing mechanism, in IFN-γ stimulated macrophages by induction of tumor necrosis factor-α. Journal of Immunology 145, 4290–4297.

Gupta, R.K., Relyveld, E.H., Lindblad, E.B., 1993. Adjuvants – a balance between toxicity and adjuvanticity. Vaccine 11, 293–306.

Holbrook, T.W., Cook, J.A., Parker, B.W., 1981. Immunization against *L. Donovani*. Glucan as an adjuvant with killed promastigotes. American Journal of Tropical Medicine and Hygiene 30, 762–768.

Howard, J.G., 1986. Immunological regulation and control of experimental leishmaniasis. International Reviews in Experimental Pathology 28, 79–116.

Iborra, S., Soto, M., Carrion, J., Nieto, A., Fernandez, E., Alonso, C., et al., 2003. The *Leishmania infantum* acidic ribosomal protein P0 administered as a DNA vaccine confers protective immunity to *Leishmania major* infection in BALB/c mice. Infection and Immunity 71, 6562–6572.

Kedzierski, L., Zhu, Y., Handman, E., 2006. *Leishmania* vaccines: progress and problems. Parasitology 133, 87–112.

Marques-da-Silva, E.A., Coelho, E.A., Gomes, D.C., Vilela, M.C., Masioli, C.Z., Tavares, C.A., 2005. Intramuscular immunization with p36 (LACK) DNA vaccine induces IFN-gamma production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge. Parasitology Research 98, 67–74.

Mauel, J., Behin, R., 1987. Immunity: clinical and experimental. In: Peters, W., Killick-Kendrick, R. (Eds.), The Leishmaniasis in Biology and Medicine, vol. 2. Academic Press, London, UK, pp. 31–739.

Mayrink, W., Williams, P., Dacosta, C.A., Magalhaes, P.A., Melo, M.N., Dias, M., et al., 1985. An experimental vaccine against American dermal leishmaniasis: experience in the state of Espirito-Santo, Brazil. Annals of Tropical Medicine and Parasitology 79, 259–269.

McElrath, M.J., Murray, H.W., Cohn, Z.A., 1988. The dynamics of granuloma formation in experimental visceral leishmaniasis. Journal of Experimental Medicine 167, 1927–1937.

Melby, P.C., Chandrasekar, B., Zhao, W., Coe, J.E., 2001. The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response. Journal of Immunology 166, 1912–1920.

Melby, P.C., Tryon, V.V., Chandrasekar, B., Freeman, G.L., 1998. Cloning of syrian hamster (*Mesocricetus auratus*) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis. Infection and Immunity 66, 2135–2142.

Mielke, M.E., Peters, C., Hahn, H., 1997. Cytokines in the induction and expression of T-cell-mediated granuloma formation and protection in the murine model of listeriosis. Immunological Reviews 158, 79–93.

Molano, I., Alonso, M.G., Miron, C., Redondo, E., Requena, J.M., Soto, M., et al., 2003. A *Leishmania infantum* multi-component antigenic protein mixed with live BCG confers protection to dogs experimentally infected with *L. Infantum*. Veterinary Immunology and Immunopathology 92, 1–13.

Murphy, M.L., Cotterell, S., Gorak, M., Engwerda, C.R., Kaye, P.M., 1998. Blockade of CTLA-4 enhances host resistance to the intracellular pathogen, *Leishmania donovani*. Journal of Immunology 161, 4153–4160.

Murray, H.W., 1990. Effect of continuous administration of interferon-γ in experimental visceral leishmaniasis. Journal of Infectious Diseases 161, 992–994.

Murray, H.W., 1999. Granulomatous inflammation: host antimicrobial defense in the tissues in visceral leishmaniasis. In: Gallin, J., Synderman, R., Fearon, D., Haynes, B., Nathan, C. (Eds.), Inflammation: Basic Principles and Clinical Correlates, third ed. Lippincott-Raven, Philadelphia, pp. 77–994.

Murray, H.W., 2001. Clinical and experimental advances in treatment of visceral leishmaniasis. Antimicrobial Agents and Chemotherapy 45, 2185–2197.

Pal, N.S., Arora, S.K., 2003. Recognition of *Leishmania donovani* by $CD4^+$ T cells of naive healthy uninfected individuals. Journal of Parasitic Diseases 27, 47–52.

Pintado, V., Martín-Rabadán, P., Rivera, M.L., Moreno, S., Bouza, E., 2001. Visceral leishmaniasis in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. A comparative study. Medicine (Baltimore) 80, 54–73.

Reed, S.G., Coler, R.N., Campos-Neto, A., 2003. Development of a leishmaniasis vaccine: the importance of MPL. Expert Reviews in Vaccines 2, 239–252.

Richards, R., Rao, M., Wassef, N., Glenn, G., Rothwell, S., Alving, C., 1998. Liposomes containing lipid A serve as an adjuvant for induction of antibody and cytotoxic T-cell responses against RTS, S malaria antigen. Infection and Immunity 66, 2859–2865.

Roach, T.I., Kiderlen, A.F., Blackwell, J.M., 1991. Role of inorganic nitrogen oxides and tumor necrosis factor α in killing *Leishmania donovani* amastigotes in γ interferon–lipopolysaccharide-activated macrophages from Lshs and Lshr congenic mouse strains. Infection and Immunity 59, 3935–3944.

Roberts, M.T., Stober, C.B., McKenzi, A.N., Blackwell, J.M., 2005. Interleukin-4 (IL-4) and IL-10 collude in vaccine failure for novel exacerbatory antigens in murine *Leishmania major* infection. Infection and Immunity 73, 7620–7628.

Sambrook, J., Russel, D.W., 2001. Molecular Cloning – A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, New York.

Sasaki, S., Tsuji, T., Hamajima, K., Fukushima, J., Ishii, N., Kaneko, T., et al., 1997. Monophosphoryl lipid A enhances both humoral and cell-mediated immune responses to DNA vaccination against human immunodeficiency virus type 1. Infection and Immunity 65, 3520–3528.

Scott, P., Pearce, E., Natovitz, P., Sher, A., 1987. Vaccination against cutaneous leishmaniasis in a murine model. 1. Induction of protective immunity with a soluble extract of promastigotes. Journal of Immunology 139, 221–227.

Shiloh, M.U., MacMicking, J.D., Nicholson, S., Brause, J.E., Potter, S., Marino, M., et al., 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity 10, 29–38.

Spitzer, N., Jardim, A., Lippert, D., Olafson, R.W., 1999. Long-term protection of mice against *Leishmania major* with a synthetic peptide vaccine. Vaccine 17, 1298–1300.

Squires, K.E., Schreibe, R.D., McElrath, M.J., Rubin, B.Y., Anderson, S.L., Murray, H.W., 1989. Experimental visceral leishmaniasis: role of endogenous IFN-γ in host defense and tissue granulomatous response. Journal of Immunology 143, 4244–4249.

Stager, S., Smith, D.F., Kaye, P.M., 2000. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. Journal of Immunology 165, 7064–7071.

Stauber, L.A., Franchino, E.M., Grun, J., 1958. An eight day method for screening compounds against *Leishmania donovani* in the golden hamster. Journal of Protozoology 5, 269–273.

Stober, C.B., Lange, U.G., Roberts, M.T., Alcami, A., Blackwell, J.M., 2005. IL-10 from regulatory T cells determines vaccine efficacy in murine *Leishmania major* infection. Journal of Immunology 175, 2517–2524.

Tewary, P., Sukumaran, B., Saxena, S., Madhubala, R., 2004. Immunostimulatory oligodeoxynucleotides are potent enhancers of protective immunity in mice immunized with recombinant ORFF leishmanial antigen. Vaccine 22, 3053–3060.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of National Academy of Sciences, USA 76, 4350–4354.

Travi, B.L., Osorio, Y., Melby, P.C., Chandrasekar, B., Arteaga, L., Saravia, N.G., 2002. Gender is a major determinant of the clinical evolution and immune response in hamsters infected with *Leishmania* spp.. Infection and Immunity 70, 2288–2296.

Ulrich, J.T., Myers, K.R., 1995. Monophosphoryl lipid A as an adjuvant. In: Powell, M.F., Newman, M.J. (Eds.), Vaccine Design: The Subunit and Adjuvant Approach. Plenum Press, New York, pp. 495–524.

Wilson, M.E., Young, B.M., Andersen, K.P., Weinstock, J.V., Metwali, A., Ali, K.M., et al., 1995. A recombinant *Leishmania chagasi* antigen that stimulates cellular immune responses in infected mice. Infection and Immunity 63, 2062–2069.

Yao, Z., Foster, P.A., Gross, G.J., 1994. Monophosphoryl lipid A protects against endotoxic shock via inhibiting neutrophil infiltration and preventing disseminated intravascular coagulation. Circulatory Shock 43, 107–114.

Zaph, C., Uzonna, J., Beverley, S.M., Scott, P., 2004. Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites. Nature Medicine 10, 1104–1110.