ELSEVIER

FEMS Immunology and Medical Microbiology 42 (2004) 241–248

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

Vaccination with *Leishmania* soluble antigen and immunostimulatory oligodeoxynucleotides induces specific immunity and protection against *Leishmania donovani* infection

Poonam Tewary, Jayesh Mehta, Bindu Sukumaran, Rentala Madhubala *

School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

Received 26 March 2004; received in revised form 27 May 2004; accepted 28 May 2004

First published online 11 June 2004

Abstract

In this report, we investigated the effect of ODN containing immunostimulatory CG motifs as adjuvant with soluble antigen (SA) from *Leishmania donovani*. BALB/c mice were vaccinated with the soluble antigen with or without CpG-ODN as adjuvant and then challenged with *L. donovani* metacyclic promastigotes. CpG-ODN alone resulted in partial protection against challenge with *L. donovani*. Immunization of mice with SA and CpG-ODN showed enhanced reduction in parasite load (~60%) when compared to SA (~40%) immunized mice. Immunization with SA by itself resulted in a mixed Th1/Th2 response whereas co-administration of SA with CpG-ODN resulted in a strong Th1 promoting isotype as they together promoted production of immunoglobulin G2a. *Leishmania*-specific Th1 cytokine response was induced by co-administering CpG-ODN and SA as they together promoted production of IFN-γ and IL-12. In the present study, we demonstrate that immunostimulatory phosphorothioate-modified ODN are promising immune enhancers for vaccination against visceral leishmaniaisis.

Keywords: Soluble antigen; CpG oligodeoxynucleotides; *L. donovani*, vaccine

1. Introduction

Leishmania are protozoan parasites that cause leishmaniasis. Worldwide 10–15 million people are infected with 2 million new cases each year and 1/10 of world population is at risk of infection [1,2]. In a recent survey, 88 countries have been declared as leishmaniasis-endemic [3,4]. *Leishmania donovani* causes the severe, often fatal disease visceral leishmaniasis (VL). The drugs that are commonly used for the treatment of VL are pentavalent antimonials. Lately there has been an increase in the cases of leishmaniasis refractory to antimonial treatment. Both human and mice develop protective immunity after recovery from VL. While the development of an anti-leishmanial vaccine appears feasible, it remains to be accomplished.

Several candidate vaccine molecules have already been evaluated in the mice model for leishmaniasis. Considerable attention and efforts toward vaccination against leishmaniasis have focused almost exclusively on localized cutaneous disease [5–10]. Purified antigens, subcellular fractions and avirulent parasites have been used as potential vaccine candidates for cutaneous leishmaniasis [11]. DNA vaccines have also been reported to induce protective immunity in a variety of experimental mouse models of infection through MHC class I and class II-restricted T-cell responses [12–14]. While a substantial progress has been made no acceptable anti-leishmanial vaccine exists against this infection. Presently there is no immunoprophylactic regimen for leishmaniasis although several human trials using

* Corresponding author. Tel./fax: +91-11-26106630.
E-mail addresses: rentala@hotmail.com, madhubala@mail.jnu.ac.in (R. Madhubala).

0928-8244/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Microbiological Societies.
doi:10.1016/j.femsim.2004.05.008

killed leishmanial promastigotes with *Mycobacterium bovis* BCG are going on and initial results look promising [15]. The mouse models of infection with the protozoan parasite *Leishmania major* have helped to define the Th1/Th2 model. Resistant C57BL/6 mice develop protective Th1 responses that control infection, whereas susceptible BALB/c mice are unable to control infection due to an aberrant Th2 response [16,17]. Treatment and vaccination that control the disease in susceptible mice invariably promote Th1 responses over Th2 responses [13,18,19]. There is no clear-cut immunological regime for *L. donovani*. In mice infected with *L. donovani*, the differential production of Th1 and Th2 does not control the cure rate, although Th1 response correlates with resistance to infection, the Th2 response does not determine susceptibility [20]. In human disease, there is a trend that both Th1 and Th2 responses against leishmaniasis coexist in humans who have been cured of visceral leishmaniasis [19,21].

Certain sequences of bacterial DNA and immunostimulatory CpG DNA can modulate immune response towards Th1 type [22–25]. The vertebrate immune system can be activated by a variety of microbial components. Among these components, CpG dinucleotides in particular base context are found in bacterial DNA. Once recognized CpG motifs activate a wide variety of innate immune responses [24,26]. This stimulatory capacity could be transferred to single-stranded oligodeoxynucleotides (ODN) containing specific sequences called as CpG-ODN. These CpG-ODN directly activate monocyte, macrophages and dendritic cells to secrete Th1-like cytokines (e.g. IL-12, TNFα and IL-6 etc.) and express increased level of cell surface co-stimulatory molecules [23,24,27–31]. These CpG-ODNs have been used as adjuvants with a range of protein vaccines and they facilitate a Th1 mediated response, where as conventional protein vaccines alone normally result in Th2 response with high titers of neutralizing antibodies and poor cellular immunity [31–38]. The potent Th1-like immune activation by CpG-ODNs suggests a possible utility for vaccination against leishmaniasis.

The role of CpG-ODN as a prophylactic vaccine adjuvant has been studied using leishmanial proteins in combination with CpG-ODN and has been reported to confer some protection after a challenge with *L. major* and *L. donovani* [37,39–41]. CpG-ODN is reported to be more potent and durable than IL-12 protein in terms of immune and biological effects in susceptible BALB/c mice after infection with *L. major* [37].

In view of the potent in vivo effects on the cellular immune response elicited by CpG-ODN, we have examined the protective efficacy of immunostimulatory DNA or CpG motifs as immune adjuvants when administered with *L. donovani* soluble antigen (SA) in a murine model system. We have also tested the effect of CpG-ODN in combination with the SA on humoral and cellular responses in BALB/c mice model of *L. donovani* infection.

2. Materials and methods

2.1. Animals

Female BALB/c mice (4–6 weeks old) were obtained from the National Institute of Nutrition, Hyderabad, India. All mice were used at ages ranging from 6 to 8 weeks. Animals were used in accordance to the institutional guidelines and the relevant committee duly approved the use of animals for this work.

2.2. Parasite culture

Leishmania donovani strain AG83 (MHOM/IN/1983/AG83) was maintained in BALB/c mice. Amastigotes were isolated from spleens, and then transformed to promastigotes in Medium 199 (Sigma, USA) supplemented with penicillin (100 U/ml Sigma, USA), 100 μg/ml streptomycin (Sigma, USA) and 30% fetal calf serum (FCS) (Biological Industries, Israel). Freshly transformed promastigotes were cultured at 22 °C in M199 medium and 10% heat-inactivated FCS and were used for infecting BALB/c mice.

2.3. Source of soluble antigen

SA was prepared from stationary phase *L. donovani* AG83 promastigotes. Cells were harvested by centrifugation at 4000*g* for 15 min at 4 °C and the pellet was washed once with phosphate buffered saline (PBS), pH 7.2. The pellet was resuspended in PBS containing protease inhibitors and lysed by sonication with six pulses of 30 s each. The lysate was centrifuged at 17,000*g* for 15 min at 4 °C and the supernatant was used as the SA [42].

2.4. Oligodeoxynucleotides

A non-CpG-ODN 1911 (5′-TCCAGGACTTTCCTCAGGTT-3′) and a CpG-ODN 1826 (5′-TCCATGA*CG*TTCCTGA*CG*TT-3′) were used in the present study. All oligonucleotides were synthesized with a nuclease resistant phosphorothioate backbone (Microsynth, Switzerland). The ODNs were resuspended in 10 mM Tris, pH 7.0/1 mM EDTA and stored at −20 °C.

2.5. Immunization of mice and assessment of parasite load

Female BALB/c mice, 6–8 weeks of age, were used for all immunizations ($n = 5$ per group). Injections were

given at midpoint of left thigh muscle. For intramuscular injection 50 μg of SA and 10 μg each of ODN in phosphate buffered saline (PBS) was used. SA was adsorbed on alum. Control mice were injected with alum diluted with PBS (final volume 100 μl). For the vaccination studies, cell proliferation, cytokine production and antibody response, mice were immunized twice at 2-week interval with alum, SA, CpG-ODN 1826 alone, SA plus CpG-ODN 1826, ODN 1911 alone and SA plus ODN 1911. Mice were bled 2 weeks following the final injection and sera from the mice in each group were pooled. For the vaccination and protection studies, mice were injected with alum, SA, CpG-ODN 1826 alone, SA plus CpG-ODN 1826 and a booster were given at 4-week interval. The mice were then challenged 2 weeks after the final boost. For the challenge experiment, 1×10^8 stationary phase promastigotes of *L. donovani* were injected intravenously via the tail vein in 100 μl of PBS per mouse.

After 4 and 8 weeks of infection, mice were euthanized and liver and spleen touch biopsies were microscopically examined after fixing and staining the slides with Giemsa. In order to quantitate levels of infection, Leishman Donovan units (LDU) were calculated as: (number of amastigotes/number of tissue nuclei) × weight of tissue in milligrams. Assessment of protection was performed using 10 mice per group. Five mice from each group were sacrificed for determination of parasitemia both at 4 and 8 weeks. The experiments were repeated twice with similar results.

2.6. Spleen cell proliferation assay

Mice were immunized twice at 2-week interval with 50 μg SA, 10 μg of each of the ODNs either alone or in combination with SA (in 100 μl of PBS). Two weeks after the final injection, spleens were removed from mice under aseptic conditions on a sterile dish containing RPMI 1640 media. Single cell suspensions were prepared by grinding the spleen with disk bottom of the plunger of a 10-ml syringe. RPMI 1640 media (5–10 ml) was added to the suspension and the contents were mixed well. The dish was kept undisturbed for 2 min and the clear supernatant was pipetted out slowly. Cells were pelleted by centrifugation at 4 °C at 250*g* (Sorvall RC-5 centrifuge, HB-4 rotor) for 10 min. Washing the pellet once with 0.9% ice-cold ammonium chloride lysed erythrocytes. The remaining cells were resuspended to a density of 2.5×10^6 cells/ml in RPMI 1640 containing 10% FCS and then divided into 200 μl aliquots (5×10^5 cells) in 96-well plates. Cells were incubated in the presence or absence of SA (5 μg/ml) and incubated for 3 days at 37 °C in an atmosphere containing 5% CO_2. Proliferation was measured by incorporation of 1 μCi of ^{3}H-thymidine over the last 16 h of the culture. Amounts of incorporation of ^{3}H were measured by liquid scintillation counter. All assays were performed in triplicate, with five mice representing each group.

2.7. Cytokine assays

The concentrations of IFN-γ, IL-4 and IL-12 in culture supernatants were determined as described previously [39]. Briefly spleen cells were isolated from mice co-injected with CpG-ODN 1826 or SA or a combination of SA and CpG-ODN 1826, ODN 1911, ODN 1911 plus SA 2 weeks after the final booster and resuspended in RPMI 1640 medium supplemented with 10% FCS. Cells were then incubated in 96-well flat bottomed plate (Nunc, Roskilde, Denmark) at a density of 5×10^5 cells per well and cultured with SA (5 μg/ml). After 48 h supernatants were collected and diluted serially, and cytokine concentrations were quantitated using enzyme linked immunosorbent assay (ELISA). The assay was performed using Opt EIA Kit (Pharmingen, SanDiego, CA) according to the manufacturer's instructions.

2.8. Isotype analysis

Serum Ig isotypes were assayed by ELISA using 100 ng of SA per well, isotype-specific secondary antibodies (biotinylated rabbit anti-mouse IgG2a, IgG2b and IgG1) and streptavidin–horseradish peroxidase (Pharmingen, San Diego, CA). In brief, 96-well plates (Immulon-4, Nunc) were coated with 100 ng of SA in 50 μl of carbonate buffer, pH 9.2, and incubated overnight at 4 °C. The plates were washed thrice with PBS containing 0.05% Tween 20 (PBST) and then blocked by incubation for 2 h at 37 °C in 200 μl of 5% milk in PBST. Plates were then washed thrice with PBST. Mouse sera (100 μl) diluted to 1:300–1:30,000 in PBST were added to the wells (except for experimental blanks which were incubated with 5% milk in PBST) and incubated at 37 °C for 2 h. The plates were washed thrice with PBST. The plates were then incubated with 100 μl of 1:2000 dilution of biotinylated antimouse IgG2a, IgG2b and IgG1 in PBST for 1 h at 37 °C and washed again. The plates were then incubated with 1:2000 of streptavidin conjugated horseradish peroxidase followed by washing thrice with PBST. Color was developed by incubating with 100 μl aliquot of *o*-phenylenediamine (5 mg/10 ml in 50 mM citrate-phosphate buffer, pH 5.2) and 10 μl of 30% H_2O_2 for 30 min. The reaction was then stopped with 50 μl of 2 N H_2SO_4. The absorbance at 490 nm was determined using a model 7520 Microplate reader (Cambridge Technology). All samples were run in triplicate. Antibody levels were determined by comparison to a standard curve generated using a pooled antiserum prepared from infected mice with high antibody titer [26,40].

2.9. Statistical analysis

Results from the different treated groups were compared by Student's t test. P values of <0.05 were considered statistically significant.

3. Results

3.1. Splenocyte proliferation in mice co-injected with SA and ODN

Splenocytes proliferation in mice injected with alum, ODN 1911, SA plus ODN 1911, CpG-ODN 1826 and CpG-ODN 1826 plus SA, was investigated in vitro as described in Section 2. Splenocytes from mice injected with ODNs did not exhibit increased proliferation of cells when compared with the SA group. However, co-administration of CpG-ODN 1826 and SA augmented splenocyte proliferation in vitro by 1.76-fold when compared to mice injected with SA alone ($P < 0.001$). Co-administration of the control ODN 1911 (which has composition matched to 1826 but lacks CpG dinucleotide motif) resulted in only 1.03-fold increase in splenocyte proliferation over the group treated with SA ($P < 0.05$) (Fig. 1). This could probably be due to a non-specifc response to the phosphorothioate background. Non-CpG-ODN has been reported to induce B cell proliferation and splenomegaly [40].

3.2. Isotype response by co-injection of ODN and SA

Antibody isotype profile provides a convenient marker of Th1 and Th2 CD^{4+} T-cell differentiation [43]. To compare IgG isotypes in non-protective vaccinated mice, sera was collected from mice 2 weeks after the second immunization and were assessed for IgG1 and IgG2a and IgG2b. Immunization with SA antigen alone gave a mixed Th1/Th2 response with more anti-SA antibodies of IgG1 (Th2) than IgG2a (Th1) isotype (Fig. 2). Control ODN without CpG motifs (1911) did not affect the isotype profile of sera from mice injected with ODN 1911 and SA antigen. In contrast, mice injected with SA antigen plus CpG-ODN 1826, the majority of antibodies were IgG2a (Fig. 2).

3.3. Cellular response to SA and ODN in vaccinated mice

Susceptible BALA/c mice were co-injected intramuscularly with CpG-ODN 1826 or SA or a combination of SA and CpG-ODN 1826, ODN 1911, ODN 1911 plus SA. Two weeks later, spleens were removed and the levels of IL-4, IL-12 and IFN-γ production in splenocytes from mice immunized with alum, ODN 1911, SA plus ODN 1911, CpG-ODN 1826 and CpG-ODN 1826 plus SA were assayed in vitro. The splenocytes were incubated in vitro in the presence SA (5 μg/ml). The cytokines levels were measured by cytokine ELISA. The levels of IFN-γ, IL-12 and IL-4 produced in splenocytes from mice immunized with soluble antigen are shown in Fig. 3(a)–(c), respectively. There was an enhanced production of IFN-γ (3.5-fold) (Fig. 3(a)) and IL-12 (2.1-fold) (Fig. 3(b)) in mice injected with SA and CpG-ODN 1826 when compared to groups where injections were given individually. However, there was no significant difference in the IL-4 levels in mice injected with either SA or CpG-ODN 1826 (Fig. 3(c)). Non-CpG-ODN did not result in enhanced production of IFN-γ (Fig. 3(a)). However, co-administration of ODN 1911

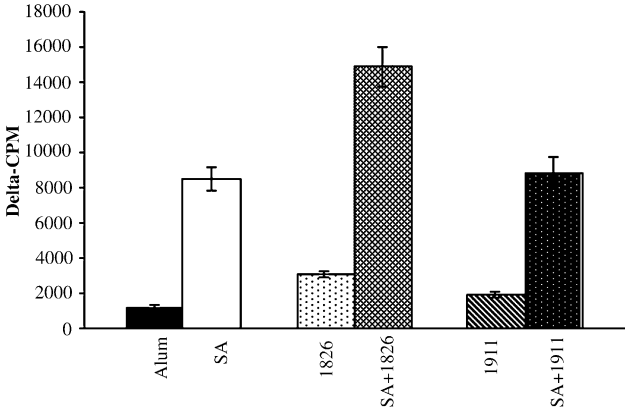

Fig. 1. Splenocyte proliferation in mice co-injected with CpG-ODN and SA. Mice were immunized twice at 2-week interval with 50 μg SA, 10 μg of each of the ODNs either alone or in combination with SA (in 100 μl of PBS). Spleens were collected as described in Section 2. Two weeks following the final injection, splenocytes were stimulated with SA and thymidine incorporation was determined. Delta-CPM represents the difference in counts compared with the corresponding non-stimulated cells.

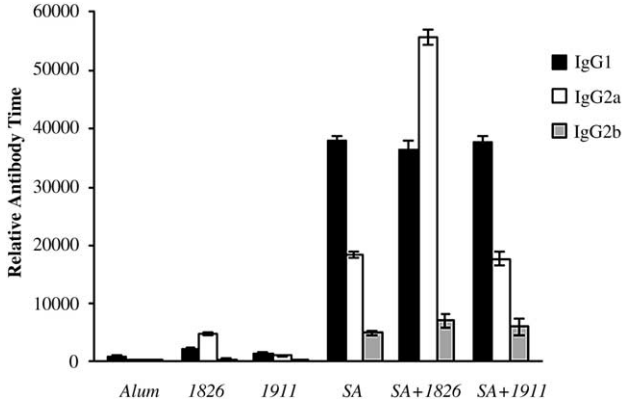

Fig. 2. Relative anti-leishmanial IgG1, IgG2a and IgG2b levels in immunized mice before challenge. Mice were bled 2 weeks after the final injection. Antibody levels are expressed relative to a standard pool as described in Section 2. Means and SE of Ig levels measured in triplicates on at least five animals per group are shown. Immunization was done using CpG-ODN 1826, ODN 1911, SA, or a combination of CpG-ODN 1826 plus SA and ODN 1911 plus SA.

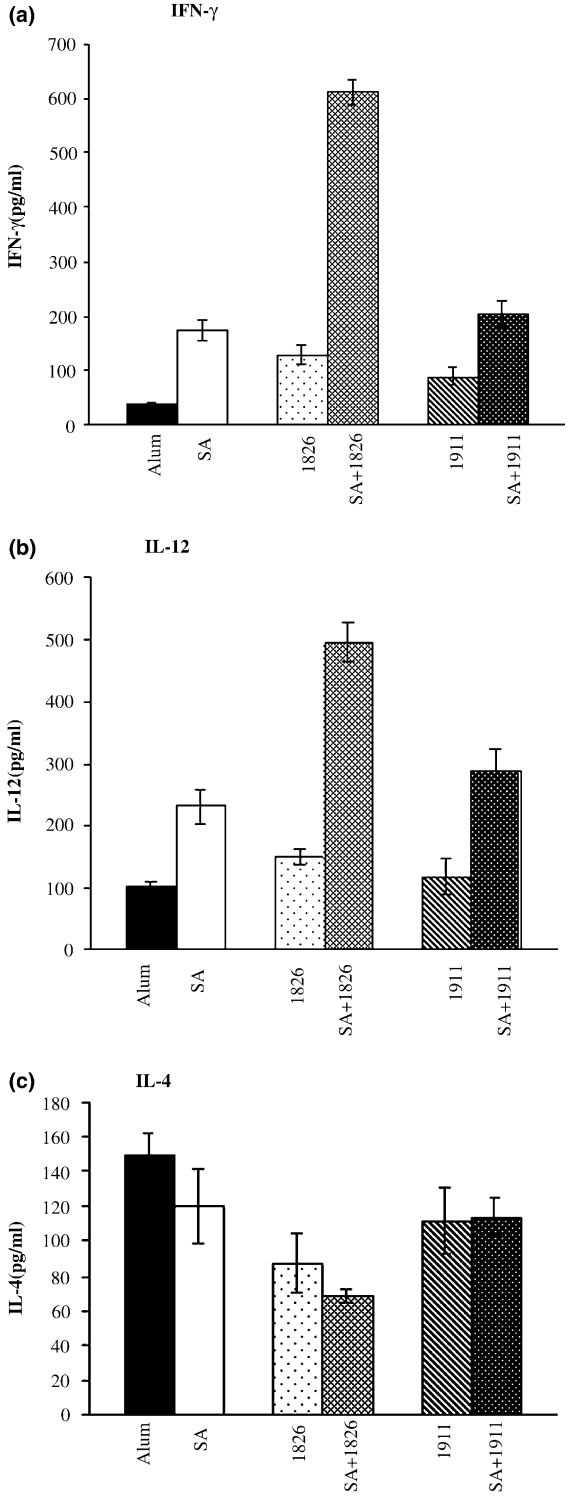

Fig. 3. Cellular immune responses in mice receiving SA and CpG-ODN 1826 and ODN 1911. BALB/c mice were immunized twice at 2-week interval with SA, 10 μg of each of the ODNs either alone or in combination with SA (in 100 μl of PBS). Spleens were collected as described in Section 2. Splenocytes were stimulated with SA and concentration of released IFN-γ, IL-12 and IL-4 in the culture supernatants was determined. Data are represented as means ± SE. Each sample was examined in triplicate and these results are representative of two experiments.

with SA showed an increase in IL-12 levels when compared to group where injection was given individually. This increase is probably due to the SA. Thus immunization with soluble antigen and CpG-ODN led to preferential production of IFN-γ and IL-12 in spleen cells stimulated with SA in vitro. It appears immunization schedule preferentially induces Th1 type immune responses. Relative antibody titers in groups immunized with SA were nearly 7659 and CpG-ODN 1826 combined with soluble antigen had a relative titer of 7968. Relative antibody titers in animals receiving ODN without CpG motifs (1911) alone or with alum were not significantly different from those in respective control groups (data not shown).

3.4. Protective immunity after immunization with CpG-ODN and Leishmania soluble antigen

Since the in vitro splenocyte proliferation and cytokine analysis data suggested that CpG-ODN 1826 resulted in IFN-γ dominant response as compared to ODN 1911, we decided to determine whether co-injection of CpG-ODN 1826 with SA would confer protective immunity. Alum by itself did not result in decreased parasite load when compared with mice injected with PBS alone. Therefore, alum was used as a control for all the experiments involving protection studies. BALB/c mice vaccinated with SA, CpG-ODN 1826 alone or SA plus CpG-ODN 1826 resulted in 42%, 33%, 55% inhibition, respectively, of parasite load in liver after 4 weeks (Fig. 4(a)). Parasite load was also checked in the spleen. The parasite load decreased by 40%, 31%, 60% in BALB/c mice vaccinated with either SA, CpG-ODN 1826 alone or SA plus CpG-ODN 1826, respectively (Fig. 4(b)). The inhibition of parasite load after 8 weeks was 40%, 35% and 59% in liver and 46%, 34% and 61% in spleen in BALB/c mice vaccinated with either SA, CpG-ODN 1826 alone or SA plus CpG-ODN 1826, respectively. A comparison of the SA vaccinated and SA plus CpG-ODN 1826 vaccinated mice shows a modifying role of the ODN on the immune response to SA.

4. Discussion

Earlier studies have demonstrated that plasmid DNA immunostimulatory sequences promote Th1 responses [32]. The ability of CpG-ODN to induce both innate and adaptive cellular immune response has made it a potential treatment and/or prophylactic vaccine adjuvant, respectively, for disease requiring cellular immunity. The strong activating effect of CpG DNA on B cells, as well as the induction of cytokines that could have indirect effects on B cells via T-helper pathway, suggests utility of CpG-ODN as a vaccine enhancer [24,26]. In the

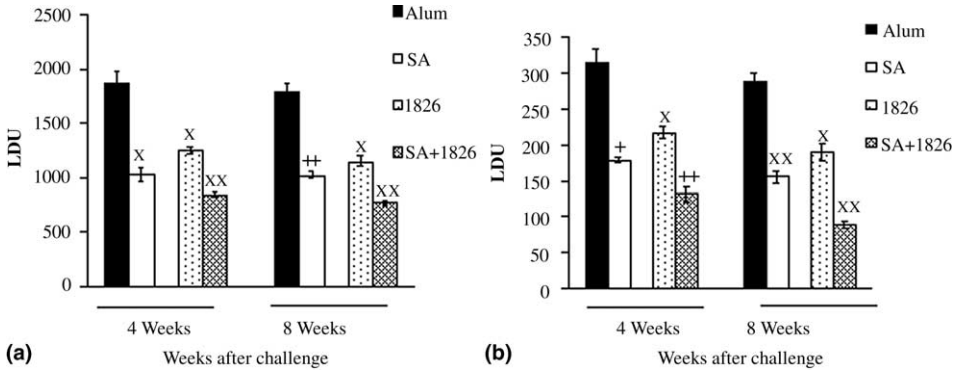

Fig. 4. Infection levels of vaccinated mice following challenge with *L. donovani*. BALB/c mice were immunized with alum, CpG-ODN 1826, SA and SA plus 1826 twice at 4-week intervals. Two weeks following the final injection, the mice were challenged intravenously with 1×10^8 *L. donovani* promastigotes. Four and 8 weeks after challenge infection, mice were killed and LDU was calculated from liver (a) and spleen smears (b). The mean LDU $\pm$ SE is shown ($n = 5$ mice per group). Means which differ significantly from that of the corresponding alum control are indicated by: +, $P < 0.005$; ++, $P < 0.0005$; ××, $P < 0.0001$ or ×, $P < 0.001$.

present study, we demonstrate that co-injection of immunostimulatory phosphorothioate-modified ODN as an adjuvant with a leishmanial SA can modulate leishmanial-specific immunity towards a protective response in mice.

This study demonstrates that the effects of immunostimulatory ODN as an adjuvant were partial and limited, as protection was only 33% in mice 4 weeks after challenge. The greater efficacy of the leishmanial soluble antigen plus CpG-ODN was observed. CpG-ODN had an in vivo impressive effect in modulating the response to vaccination with soluble antigen. Administration of SA alone or with CpG-ODN resulted in ~40% and ~60% reduction in the parasite load in liver at 8 weeks after challenge, respectively.

Earlier observations have also shown that susceptible BALB/c mice when immunized with *Leishmania* SA with or without CpG-ODN as adjuvant and then challenged with *L. major* metacyclic promastigotes showed a highly significant reduction in swelling when compared to SA-vaccinated mice and enhanced survival when compared to unvaccinated mice [39,40]. CpG-ODN, when used as a vaccine adjuvant with either a recombinant protein or heat killed leishmanial antigen, can induce long-term protection against an intracellular infection in a CD^{8+}-dependent manner [37]. Individual mice immunized with ODNs and *Leishmania* lysate demonstrated *Leishmania*-specific lymphocyte proliferation and IFN-γ protection in response to parasites in vitro [39]. The mechanism by which CpG-ODN mediate their function in vivo is by enhancing antigen presenting cells (APC) function through toll-like receptor 9, thereby augmenting both the activation and maturation of dendritic cells (DC) as well as the induction of proinflammatory cytokines [43]. CpG-ODN induction of IL-12, IL-18 and other soluble mediators from activated DC is likely to result in a more physiological cognate interaction between the DC and T-cells, resulting in both a qualitative and quantitatively different type of CD^{4+} and CD^{8+} T-cell response [37].

It is well established that CpG-ODN are potential inducers of Th1 responses, which is consistent with our findings that mice vaccinated with ODN plus SA had striking enhancement of the production of IFN-γ and IL-12. Use of CpG-ODN along with SA resulted in almost additive effect in the production of cytokines in vitro. Immunization with SA antigen alone gave a mixed Th1/Th2 response with more anti-SA antibodies of IgG1 (Th2) than IgG2a (Th1) isotype. In contrast, in mice injected with SA antigen with CpG-ODN 1826, the majority of antibodies were IgG2a.

Thus, the immunostimulatory effect of CpG-ODN can be utilized to create a protective vaccine when given in combination with specific antigen from *L. donovani* in mice model system. Our results in murine *L. donovani* model, shows promising role of ODN as adjuvants in combination with *Leishmania*-specific antigen and can be exploited to create a protective vaccine. Additionally, CpG-ODN may be very useful for eliciting an enhanced cellular and humoral response and increasing the efficacy of individual antigens that induce partial but incomplete protection. Further studies would be required to evaluate the effect of different doses of CpG-ODN to further improve the efficacy of the present vaccine for visceral leishmaniasis in a murine model.

Acknowledgements

This work is supported by the Life Science Research Board Grant from the Defense Research and Development Organization, Government of India, New Delhi, India to R.M.B., P.T. and B.S. are supported by a grant from the University Grants Commission, New Delhi, India.

References

[1] Baily, G.G. and Nandy, A. (1994) Visceral leishmaniasis: more prevalent and more problematic. J. Infect. 29, 241–247.

[2] Herwaldt, B.L. (1999) Leishmaniasis. Lancet 354, 1191–1199.

[3] World Health Report. Thirteenth Programme Report (1997) Special Programme for Research and Training in Tropical Diseases. Leishmaniasis. World Health Organizations Publications, Geneva.

[4] Advances in battle against leishmaniasis. (1998) TDR News. 57, 2.

[5] Alexander, J. (1982) A radioattenuated *Leishmania major* vaccine markedly increases the resistance of CBA mice to subsequent infection with *Leishmania mexicana mexicana*. Trans. R. Soc. Trop. Med. Hyg. 76, 646–649.

[6] Campos-Neto, A., Porrozzi, R., Greeson, K., Coler, R.N., Webb, J.R., Seiky, Y.A., Reed, S.G. and Grimaldi Jr., G. (2001) Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. Infect. Immun. 69, 4103–4108.

[7] Howard, J.G., Nicklin, S., Hale, C. and Liew, F.Y. (1982) Prophylactic immunization against experimental leishmaniasis: I. Protection induced in mice genetically vulnerable to fatal *Leishmania tropica* infection. J. Immunol. 129, 2206–2212.

[8] Howard, J.G., Liew, F.Y., Hale, C. and Nicklin, S. (1984) Prophylactic immunization against experimental leishmaniasis. II. Further characterization of the protective immunity against fatal *Leishmania tropica* infection induced by irradiated promastigotes. J. Immunol. 132, 450–455.

[9] Mayrink, W., da Costa, C.A., Magalhaes, P.A., Melo, M.N., Dias, M., Lima, A.O., Michalick, M.S. and Williams, P. (1979) A field trial of a vaccine against American dermal leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 73, 385–387.

[10] Mayrink, W., Antunes, C.M., da Costa, C.A., Melo, M.N., Dias, M., Michalick, M.S., Magalhaes, P.A., De Oliveira Lima, A. and Williams, P. (1986) Further trials of a vaccine against American cutaneous leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 80, 1001.

[11] Handman, E. (2001) Leishmaniasis: current status of vaccine development. Clin. Microbiol. Rev. 14, 229–243.

[12] Gurunathan, S., Sacks, D.L., Brown, D.R., Reiner, S.L., Charest, H., Glaichenhaus, N. and Seder, R.A. (1997) Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. J. Exp. Med. 186, 1137–1147.

[13] Sukumaran, B., Tewary, P., Saxena, S. and Madhubala, R. (2003) Vaccination with DNA encoding ORFF antigen confers protective immunity in mice infected with *Leishmania donovani*. Vaccine 21, 1292–1299.

[14] Sjolander, A., Baldwin, T.M., Curtis, J.M. and Handman, E. (1998) Induction of a Th1 immune response and simultaneous lack of activation of a Th2 response are required for generation of immunity to leishmaniasis. J. Immunol. 160, 3949–3957.

[15] Cabrera, M., Blackwell, J.M., Castes, M., Trujillo, D., Convit, J. and Shaw, M.A. (2000) Immunotherapy with live BCG plus heat killed *Leishmania* induces a T helper 1-like response in American cutaneous leishmaniasis patients. Parasite Immunol. 22, 73–79.

[16] Julia, V., Rassoulzadegan, M. and Glaichenhaus, N. (1996) Resistance to *Leishmania major* induced by tolerance to a single antigen. Science 274, 421–423.

[17] Launois, P., Maillard, I., Pingel, S., Swihart, K.G., Xenarios, I., Acha-Orbea, H., Diggelmann, H., Locksley, R.M., MacDonald, H.R. and Louis, J.A. (1997) IL-4 rapidly produced by V β4 V alpha8 CD^{4+} T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. Immunity 6, 541–549.

[18] Diego Miralles, G., Stoeckle, M.Y., McDermott, D.F., Finelman, F.D. and Murray, H.W. (1994) Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. Infect. Immun. 62, 1058–1063.

[19] Kemp, M., Kurtzhal, J.A., Bendtzen, K., Poulsen, L.K., Hansen, M.B., Koech, D.K., Kharazmi, A. and Theander, T.G. (1993) *Leishmania donovani*-reactive Th1- and Th2-like T-cell clones from individuals who have recovered from visceral leishmaniasis. Infect. Immun. 61, 1069–1073.

[20] Kaye, P.M., Curry, A.J. and Blackwell, J.M. (1991) Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis. J. Immunol. 146, 2763–2770.

[21] Karp, C.L., el-Safi, S.H., Wynn, T.A., Satti, M.M., Kordofani, A.M., Hashim, F.A., Hag-Ali, M., Neva, F.A., Nutman, T.B. and Sacks, D.L. (1993) In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-γ. J. Clin. Invest. 91, 1644–1648.

[22] Halpern, M.D., Kurlander, R.J. and Pisetsky, D.S. (1996) Bacterial DNA induces murine interferon-γ production by stimulation of interleukin-12 and tumor necrosis factor-α. Cell. Immunol. 167, 72–78.

[23] Klinman, D.M., Yi, A.K., Beaucage, S.L., Conover, J. and Krieg, A.M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ. Proc. Natl. Acad. Sci. USA 93, 2879–2883.

[24] Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A. and Klinman, D.M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374, 546–549.

[25] Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.D., Silverman, G.J., Lotz, M., Carson, D.A. and Raz, E. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 273, 352–354.

[26] Klinman, D.M., Barnhart, K.M. and Conover, J. (1999) CpG motifs as immune adjuvants. Vaccine 17, 19–25.

[27] Ballas, Z.K., Rasmussen, W.L. and Krieg, A.M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol. 157, 1840–1845.

[28] Cowdery, J.S., Chace, J.H., Yi, A.K. and Krieg, A.M. (1996) Bacterial DNA induces NK cells to produce IFN-γ in vivo and increases the toxicity of lipopolysaccharides. J. Immunol. 156, 4570–4575.

[29] Messina, J.P., Gilkeson, G.S. and Pisetsky, D.S. (1991) Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. J. Immunol. 147, 1759–1764.

[30] Tokunaga, T., Yamamoto, H., Shimada, S., Abe, H., Fukuda, T., Fujisawa, Y., Furutani, Y., Yano, O., Kataoka, T. and Sudo, T., et al. (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. J. Natl. Cancer Inst. 72, 955–962.

[31] Weiner, G.J., Liu, H.M., Wooldridge, J.E., Dahle, C.E. and Krieg, A.M. (1997) Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc. Natl. Acad. Sci. USA 94, 10833–10837.

[32] Chu, R.S., Targoni, O.S., Krieg, A.M., Lehmann, P.V. and Harding, C.V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J. Exp. Med. 186, 1623–1631.

[33] Davis, H.L., Weeratna, R., Waldschmidt, T.J., Tygrett, L., Schorr, J., Krieg, A.M. and Weeranta, R. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. 160, 870–876.

[34] Lipford, G.B., Bauer, M., Blank, C., Reiter, R., Wagner, H. and Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur. J. Immunol. 27, 2340–2344.

[35] Roman, M., Martin-Orozco, E., Goodman, J.S., Nguyen, M.D., Sato, Y., Ronaghy, A., Kornbluth, R.S., Richman, D.D., Carson, D.A. and Raz, E. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat. Med. 3, 849–854.

[36] Sun, S., Kishimoto, H. and Sprent, J. (1998) DNA as an adjuvant: capacity of insect DNA and synthetic oligodeoxynucleotides to augment T cell responses to specific antigen. J. Exp. Med. 187, 1145–1150.

[37] Rhee, E.G., Mendez, S., Shah, J.A., Wu, C.Y., Kirman, J.R., Turon, T.N., Davey, D.F., Davis, H., Klinman, D.M., Coler, R.N., Sacks, D.L. and Seder, R.A. (2002) Vaccination with heat-killed leishmania antigen or recombinant leishmanial protein and CpG oligodeoxynucleotides induces long-term memory CD^{4+} and CD^{8+} T cell responses and protection against *Leishmania major* infection. J. Exp. Med. 195, 1565–1573.

[38] Zimmermann, S., Egeter, O., Hausmann, S., Lipford, G.B., Rocken, M., Wagner, H. and Heeg, K. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. J. Immunol. 160, 3627–3630.

[39] Walker, P.S., Scharton-Kersten, T., Krieg, A.M., Love-Homan, L., Rowton, E.D., Udey, M.C. and Vogel, J.C. (1999) Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN-γ-dependent mechanisms. Proc. Natl. Acad. Sci. USA 96, 6970–6975.

[40] Stacey, K.J. and Blackwell, J.M. (1999) Immunostimulatory DNA as an adjuvant in vaccination against *Leishmania major*. Infect. Immun. 67, 3719–3726.

[41] Poonam, T., Sukumaran, B., Saxena, S., Madhubala, R. (2004) Immunostimulatory oligodeoxynucleotides are potent enhancers of specific immunity in mice immunized with recombinant ORFF leishmanial antigen vaccine (in press).

[42] Dole, V.S., Raj, V.S., Ghosh, A., Madhubala, R., Myler, P.J. and Stuart, K.D. (2000) Immunization with recombinant LD1 antigens protects against experimental leishmaniasis. Vaccine 19, 423–430.

[43] Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A toll-like receptor recognizes bacterial DNA. Nature 408, 740–745, Erratum in: Nature 2001. 409:646.