Kinesin Motor Domain of *Leishmania donovani* as a Future Vaccine Candidate

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Visceral leishmaniasis (VL) is one of the important parasitic diseases, with approximately 350 million people at risk. Due to the nonavailability of an ideal drug, development of a safe, effective, and affordable vaccine could be a solution for control and prevention of this disease. The present study was carried out to examine the immunological potential of kinesin protein from the microtubule locus of *Leishmania donovani* as a suitable vaccine candidate. In silico analysis of this region revealed clusters of major histocompatibility complex class I and II binding epitopes in its motor domain region. A recombinant protein was expressed from this region and named rLvacc. The antigenicity and immunogenicity studies of this protein by Western blot analysis revealed that rLvacc is strongly recognized by sera from acute VL patients. To evaluate its immunogenicity, peripheral blood mononuclear cells from cured VL patients were separated, and a lymphocyte proliferation assay was carried out in the presence of rLvacc. After lymphocyte proliferation, the pooled culture supernatant was assayed for anti-rLvacc antibody titers using an enzyme-linked immunosorbent assay. The results showed that immunoglobulin G2 (IgG2) subtype antibodies were predominant, while IgG1 subtype antibodies were produced in very low titers. On the basis of these ex vivo preliminary findings, its immunogenicity was studied in BALB/c mice. Vaccination with the DNA construct generated a good cellular immune response with significant increases in gamma interferon and interleukin-2 (IL-2) cytokine levels (Th1), but no increase in IL-4 levels (Th2). Taken together, our findings suggest the kinesin motor domain region of *L. donovani* as a potential vaccine candidate against visceral leishmaniasis.

Visceral leishmaniasis (VL) is a major public health problem in tropical and subtropical countries, such as India, Bangladesh, Nepal, Sudan, and Brazil. The disease is caused by an intracellular protozoan parasite of the *Leishmania donovani*/*L. infantum*/*L. chagasi* complex. Visceral leishmaniasis is characterized by fever, hepatosplenomegaly, cachexia, pancytopenia, and hypergammaglobulinemia. The disease is fatal if left untreated. The characteristic immunological feature of active VL is absence of parasite-specific cell-mediated immune responses (16). It has been reported that there is an increase in interleukin-4-positive (IL-4+) neutrophils, natural killer (NK) cells, and IL-10+ monocytes, while the number of gamma interferon-positive (IFN-γ+), IL-2+, and IL-12+ eosinophils is significantly decreased. However, serum levels of tumor necrosis factor alpha and IL-6 were high in patients with active visceral leishmaniasis (5, 19, 24). In contrast, the cured cases present a characteristic type 1 response with an increase in IFN-γ+, IL-2+, neutrophils, eosinophils, and NK cells and with an increase in IL-12+ monocytes (19, 21, 24). Asymptomatic cases present a mixed profile, characterized by an increase in IFN-γ+, IL-2+ neutrophils, eosinophils, and NK cells and IL-12+ eosinophils and monocytes, as well as an increase in IL-4+ neutrophils and IL-10+ eosinophils and monocytes (19, 24).

Helper and cytotoxic T cells are known to play an integral role in the immune response to this infection, connecting the innate immune response to the development of efficient adaptive cellular immunity, mainly through IL-2 and IFN-γ production. These two cytokines drive the effector functions of macrophages and trigger a Th1 immune response (24). These findings suggest that any intervention that helps the shift of the immune response from Th2 type toward Th1 type will have a major role in cure and prevention of visceral leishmaniasis.

Therefore, modalities to immunopotentiate the Th1 arm of the immune response could be exploited as vaccine candidates. In this direction several studies have reported various genes and their proteins as suitable vaccine candidates against this disease. The proteins reported include gp 63, leishmania-activated C kinase (LACK), A2, and hydrophilic acylated surface protein B1 (13, 14, 25, 29). However, the major problem with most of these candidates was immunogenicity and the level of protection they conferred. The protective efficacy of purified gp63 has been tested in several experimental models using different challenge strains and adjuvants. However, the studies revealed its poor protective efficacy. Apart from gp63, other antigens like LACK and A2 were also tested, but the results were not satisfactory (13, 14, 25, 29). Failure of these single-antigen vaccines led to development of conjugate polypeptide vaccines. One such candidate is a recombinant polypeptide comprising a tandem fusion of the leishmanial antigens thiolspecific antioxidant, *L. major* stress-inducible protein 1, and leishmania elongation initiation factor delivered with mono-phosphoryl lipid A-squalene suitable for human use. This vaccine candidate is the first defined vaccine for cutaneous leishmaniasis in human clinical trials and has completed phase 1

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and 2 safety and immunogenicity testing in normal, healthy human subjects. The vaccine candidate also showed good protective efficacy against visceral leishmaniasis in a hamster model (8, 9).

Besides recombinant antigens, naked DNA vaccines have become popular recently and have revolutionized prevention and treatment strategies against infectious diseases. However, DNA vaccines have not made much in-road in the field of medical parasitology, except against malaria, to some extent. Work on a DNA vaccine against Leishmania donovani is in progress. Vaccination with the ORF gene induced both humoral and cellular immune responses against ORFF, which provided a significant level of protection against challenge with L. donovani in a mouse model (30). Similarly, a DNA vaccine based on kinetoplastid membrane protein 11 provided protection to golden hamsters against both antimony-responsive and -resistant Leishmania strains developed (2). However, further studies involving efficacy studies in primate models or clinical trials need to be done prior to their application in humans.

Recently, our laboratory has completely characterized the kinesin gene from two Indian isolates of L. donovani (GenBank accession no. AY615886 and AY615887). The sequencing results revealed that the kinesin gene is ~3,000 bp long, comprised of one long open reading frame (the kinesin motor domain) followed by an immunodominant repeat region with four to six tandem repeats of 117 bp. The protein expressed by the immunodominant repeat region is highly sensitive and specific for the diagnosis of VL and post-kala-azar dermal leishmaniasis patients and was named rKE16 (28). The kinesin region of Leishmania donovani expressed by the motor domain is a member of the kinesin protein superfamily (KIF). These proteins are conserved and responsible for ATP hydrolysis and microtubule binding. In Leishmania donovani, it has been found to play an important role in cell division and intracellular transport of various cargos, including vesicles, organelles, and large protein complexes, cytoskeletal filament formation, and flagellar beating. The protein is highly conserved in nature, at both the nucleotide sequence and amino acid sequence levels, demonstrating 80 to 90% identity with other Leishmania species and 35 to 45% identity in organisms such as trypanosomes, mice, and humans, (3, 18, 28). The present study was carried out to evaluate its ability to stimulate a cellular response in the lymphocytes of cured patients of visceral leishmaniasis and in a BALB/c mouse model, with the possibility of exploiting it as a DNA vaccine candidate.

MATERIALS AND METHODS

In silico analysis of the kinesin peptide sequence. To determine the immunogenic domains in the kinesin protein of L. donovani, we carried out in silico analysis of the kinesin peptide sequence using the two promiscuous major histocompatibility complex (MHC) class I and II binding peptide prediction servers (available at http://www.imtech.res.in/raghava and http://www.jenner.ac.uk/MHCpred) (27). These servers predict the specific epitopes by using a neural network and physicochemical properties of the epitopes. Our analysis revealed clusters of MHC-I and -II binding epitopes with high affinity for cytotoxic and helper T cells in the kinesin motor domain region. Further amino acid sequence analysis revealed the kinesin motor domain region in the Leishmania major, Leishmania infantum, and Leishmania braziliensis proteome also. The kinesin motor domain of Leishmania donovani KE-16 (AAT40474) shares 94% homology with L. major strain Friedlin (CAO3170), 83% homology with L. infantum JPCM5 (CAM66680), and 77% homology with L. braziliensis HMOM/BR/75/M2904 (CAM37580). Moreover, we were able to identify MHC binding epitopic regions in kinesin motor domain of these strains also. There is homology in the epitopic regions identified in the L. major strain Friedlin amino acid sequence. However, there is much heterogeneity in the epitopic regions identified in L. infantum JPCM5 and L. braziliensis HMOM/BR/75/M2904. This analysis reveals that the kinesin motor domain may be a promising leishmania vaccine candidate.

On the basis of these in silico analyses, primers capable of amplifying the open reading frame of the kinesin motor domain (bp 361 to 1350) of Leishmania donovani were designed with sequences for the sense primer 5′-CCA ATG CAT TGG ATG ACC CAC CCT TCC ACT GTG CGG-3′ and antisense primer 5′-GGG CCC GTC GAT CAC GGG CCC GAG CGT CGT-3′. Using these primers, the kinesin motor domain region was amplified from the KE-16 strain of Leishmania donovani (GenBank accession no. AY615886). The amplified product was isolated, purified, and cloned in the pGEM-T vector, and later it was sequenced (Microsynth, Switzerland) to verify the insertion.

Expression and Western blot analysis of rLvacc using VL patient serum. rLvacc was expressed as an N-terminal histidine-tagged (six-His) protein in the pPRSET-C vector (Invitrogen, San Diego, CA) and purified twice to homogeneity by affinity chromatography on a Ni-nitrotriacetic acid (NTA) resin column (Qiagen, Chatsworth, CA), following a standard protocol as described earlier (28). Briefly, E. coli cells BL21(DE3) logarithmic-phase cells were transformed with the pPRSET-C plasmid and induced for 4 h with 1 mM isopropyl-β-D-thiogalactoside. Following induction, the bacterial cells were lysed and the recombinant proteins were purified using a Ni-NTA agarose column (Qiagen) under native conditions following the manufacturer’s protocol. The lysate was centrifuged at 10,000 × g for 30 min at 4°C to sediment the cellular debris. The supernatant was aspirated, and an aliquot of 50 μl was stored at 2°C for records and controls. One milliliter of 50% Ni-NTA slurry was added to 4 ml of clarified and mixed and incubated at 4°C for 60 min. The lysate-Ni-NTA mixture was then loaded onto a column with the bottom outlet capped. The flowthrough was collected after removing the bottom cap, the column was washed twice with 4 ml of wash buffer, and the fractions were collected for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The protein was eluted four times with 0.5 ml of imidazole buffer, and the eluate was collected in four tubes. The optimal imidazole concentration for protein elution from nicked column was determined by eluting initially with increasing binding concentration of imidazole viz., 40, 80, 160, and 240 mM, and analyzing the fractions by 12% SDS-PAGE. The recombinant proteins were optimally eluted at a 160 mM imidazole concentration. The eluted protein was then finally concentrated, and small protein contaminants (<10 kDa) were removed by size exclusion centrifugation (Amicon, Beverly, MA). rLvacc was subsequently purified, and possible lipopolysaccharide contamination was also removed with a polysaccharin B-garose column (Qiagen, Chatsworth, CA). The molecular mass of the 361-amino-acid-long rLvacc peptide was estimated to be 35 kDa by molecular mass prediction software (Protein calculator v3.2; http://www.scripps.edu/~cdpustam/protcalc.html).

In order to check the presence of antibodies against rLvacc in VL patient serum, Western blot analysis was carried out. The purified rLvacc protein was retarded on 12% SDS-PAGE and transferred onto nitrocellulose filters (Millipore, MA) using a semidry blotting apparatus (Bio-Rad) following the manufacturer’s protocol. The membrane was blocked with 5% skimmed milk, washed thrice with phosphate-buffered saline (PBS) with 0.1% Tween 20, pH 7.0 (PBS-T) for 10 min each, and incubated thereafter with patient serum samples (1:50 dilution in PBS, pH 7.0, 1% bovine serum albumin [BSA]) for 1 h at room temperature. The membrane was washed thrice with PBS-T and incubated for 1 h at room temperature with a 1:1,000 dilution of biotin-conjugated anti-human immunoglobulin G (IgG; in PBS, pH 7.0, 1% BSA). The membrane was incubated with secondary antibody, washed, and incubated with primary substrate avidine-conjugated to horseradish peroxidase. After washing thrice for 10 min each with PBS, pH 7.0, 0.1% Tween 20, the blots were developed using diaminobenzidine (DAB; Amresco, OH) and 0.1% H2O2 as substrate, and bands were visualized by the naked eye.

Induction study and cell proliferation assay. After institutional ethical committee approval, 10 serologically and parasitologically confirmed and patients successfully treated for kala-azar (all male, with ages ranging from 19 to 48 years [29 ± 8 years, mean ± standard deviation]) were included in the study. Five healthy controls (24 to 45 years old [31 ± 8]) from nonendemic regions were also included. All kala-azar patients hailed from endemic rural areas of Bihar, Eastern India. From these patients, 10 ml heparinized blood was collected in sterile tubes and peripheral blood mononuclear cells (PBMCs) were separated using density gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). PBMCs from five nonendemic healthy controls were also separated using the same protocol. Mononuclear cells were resuspended in RPMI 1640 (HiMedia, India) supplemented with 25 mM HEPES, 0.2% sodium bicarbonate, and
10% fetal calf serum. To the medium, 100 units each of penicillin and streptomycin dissolved in 100 µl of sterile PBS were added. These cells (2 × 10^5) were distributed in triplicate in 96-well round-bottom microtiter plates (TPP, Switzerland) in a final volume of 200 µl. Cultures were stimulated either with rLvacc (5 µg/well) or concanavalin A (1 µg/well) for 72 h, and cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) as described elsewhere, with minor modifications (22). In brief, MTT solution (10 µl, 5 mg/ml) was added to 200 µl of medium and incubated for 3 h at 37°C. After incubation the culture medium was removed and dark blue formazan crystals were dissolved in acidic isopropanol (100 µl of 0.04 N HCl in isopropanol). Color formation was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Biotek Instruments). Wells with concanavalin A served as positive controls, while wells without any stimulation served as negative controls.

**Immune response against the expressed protein.** The immunogenicity of rLvacc was measured by ELISA as described earlier (12). In brief, a 96-well ELISA plate (Dynatech) was coated with rLvacc at a concentration of 100 ng/well overnight in bicarbonate buffer (pH 9.2) at 4°C, and the next morning it was washed three times with the washing buffer PBS-T (PBS with Tween 20, pH 7.2) in an automated ELISA plate washer (Adalantis, Austria). The wells were blocked with 1% BSA in PBS (pH 7.2) and incubated for 1 h at room temperature followed by washing the plate again three times with PBS-T. The PBMC culture supernatants were aspirated and diluted 1:50 in PBS, and 200 µl of diluted supernatant was added to each antigen-coated well and incubated at 37°C for 1 h. After 1 h, the plate was again washed in the automated ELISA plate washer, followed by addition of 100 µl of anti-human IgG1- and IgG2-biotin conjugate (Sigma-Aldrich, St. Louis, MO) in each well (including control wells), and again incubated for 30 min at room temperature, followed by washing the plate again three times under the same conditions as mentioned above, followed by addition of 50 µl of chromogenic substrate to each well. The reaction was stopped after 0.5 hour by addition of 1 N H2SO4 solution, and absorbance was measured at 450 nm using an ELISA plate reader (Biotek Instruments). Each test was performed with appropriate positive and negative controls. The mean optical density (OD) values of nonendemic healthy controls + 2 standard deviations were taken as the cutoff.

**Generation of the kinesin motor domain DNA vaccine construct.** The DNA vaccine expressing the kinesin motor domain of Leishmania donovani was prepared by restriction digestion of the kinesin motor domain gene (993 bp) from a pGEM-T vector using BstXI and ApaI restriction enzymes and cloned into the mammalian expression vector pVAX1 (Invitrogen). This vector is a shuttle vector that can be screened easily using Escherichia coli, and it also has a cytomegalovirus promoter for high expression levels in a wide range of mammalian cells, with a kanamycin resistance gene as a selection marker.

For generating the DNA vaccine construct, the fragment was digested, purified, and ligated cohesively in the vector by using T4 DNA ligase. After ligation, the transcription-deficient and endonuclease-deficient E. coli DH5α strain was transformed using a pVAX1-kinesin motor domain construct. All the recombinant molecules generated were screened using antibiotic pressure selection. Finally, the plasmid DNA was purified from the recombinant clones. Apart from the kinesin motor domain construct, a DNA vaccine expressing a 468-bp kinesin immunodominant repeat construct was also generated and included as control.

**In vitro expression analysis of clones.** The ability of the recombinant construct to express the kinesin motor domain was studied in vitro using a transcription and translation (TNT)-coupled system (Promega) according to the manufacturer’s instruction. TNT uses a coupled transcription/translation reaction for in vitro protein synthesis. Transcription and translation take place simultaneously in the reaction; while the RNA polymerase transcribes the template gene, the ribosomes provided by the TNT start to translate the 5’ end of nascent mRNA. To confirm the expression, Western blot analysis was carried out with kala-azar patient sera using a standard methodology, as described above.

**Immunogenicity study of the DNA vaccine construct.** Six BALB/c mice, 6 to 8 weeks of age, were used in each group for all immunization studies. For intramuscular vaccination, 100 µg of plasmid DNA (in 50 µl of PBS) was injected into the midpoint of the left thigh muscle. The mice were immunized twice at a fortnightly interval. Another set of six mice injected with empty plasmid served as controls. Another set of six mice was also included as a control and was vaccinated with the kinesin immunodominant repeat domain (nonmotor) construct, which is predominantly a B-cell epitopic region. The injected mice were bled and sacrificed after 2 weeks of final injections. Spleens were removed from mice under aseptic conditions on a sterile dish containing RPMI 1640 medium. Single-cell suspensions were prepared by grinding the spleen with the disk bot-

tom of the plunger of a 10-ml syringe. To the suspension, 10 ml of RPMI 1640 medium was added and mixed to homogeneity. The dish containing cell suspension was kept undisturbed for 2 min, and the clear supernatant was pipetted out slowly. Cells were pelleted by centrifuging at 4°C at 250 × g (Sigma 2K centrifuge) for 10 min. The pellet containing erythrocytes and spleenocytes was collected and washed once with 0.9% ammonium chloride to lyse erythrocytes. The remaining cells (splenocytes) were resuspended to a density of 2.5 × 10^6 cells/ml in RPMI 1640 containing 10% fetal calf serum and 0.05 µM 2-mercaptoethanol. The cell suspension was then distributed as 200-µl aliquots (5 × 10^5 cells) in 96-well plates. After addition of the mitogen concanavalin A (1 µg/well), rLvacc (1 µg/well), or rKe16 (1 µg/well), the cells were incubated for 3 days at 37°C in an atmosphere containing 5% CO2 and 95% humidity (30). Proliferation was measured in an MTT assay (22).

IFN-γ, IL-2, and IL-4 detection in spleenocyte cultures of mice in response to vaccination. The spleenocyte cultures (isolated from mouse spleens) were set up in 96-well culture plates at a concentration of 5 × 10^5 cells/well, followed by addition of antigens/mitogens, with each group set up in triplicate. After 48 h, the supernatants were harvested carefully from each well for cytokine measurements. IFN-γ, IL-2, and IL-4 in the supernatants were determined by using an Endogen mouse IFN-γ, IL-2, and IL-4 enzyme-linked immunosorbent assay kit (Pierce Biotechnology, Inc.) following the protocol recommended by the manufacturer.

**Immunoproliferative response to whole parasite.** The present study was carried out to check immune reactivation to whole parasite following vaccination. For this, we isolated spleenocytes from kinesin motor domain construct-vaccinated mice and carried out a spleenocyte proliferation assay in the presence of whole-cell lysate (1 µg/well) and further estimated the cytokine profile using the protocol described for the protein described, followed by washing 100 µl of avidin-biotin horseradish peroxidase (HRP) conjugate was added to each well, and the plate was incubated for 30 min at room temperature. Finally, the plate was again washed three times under the same conditions as mentioned above, followed by addition of 50 µl of chromogenic substrate to each well. The reaction was stopped after 0.5 hour by addition of 1 N H2SO4 solution, and absorbance was measured at 450 nm using an ELISA plate reader (Biotek Instruments). Each test was performed with appropriate positive and negative controls. The mean optical density (OD) values of nonendemic healthy controls + 2 standard deviations were taken as the cutoff.

**RESULTS**

rLvacc showed strong immunoreactivity with sera from cured VL patients. The cloning and expression of the kinesin motor domain in pRSET-C resulted in high-level expression of the rLvacc protein with a molecular mass of 35 kDa (after removal of the His tag). We obtained an expression level of 2.6 mg of purified recombinant protein from a 1-liter culture and a purity of >96%. The DNA content of the purified protein sample was approximately ≈9 ng/ml, which is well within the WHO recommendations on biological standardization for recombinant proteins. While assessing the antibody response against the recombinant rLvacc, the sera from VL patients reacted with rLvacc in Western blotting (Fig. 1). Sera from healthy controls did not react with rLvacc at all.

**rLvacc stimulation induced a strong proliferative response in PBMCs from cured VL patients.** The results of the proliferative response of PBMCs against rLvacc at a 5-µg/well concentration showed significantly higher stimulation in cured kala-azar patients (mean OD, 0.4628 ± 0.014) (Fig. 2) than nonendemic healthy controls (mean OD, 0.1046 ± 0.003) (Fig. 2). The difference was statistically significant (P < 0.0001). Concanavalin A controls showed a good proliferative response in both cured kala-azar patients and healthy controls, indicating the procedural sensitivity as shown in Fig. 2.

**rLvacc stimulation induced strong IgG2 production in PBMCs from VL patients.** Supernatants from the rLvacc-stimulated PBMCs of cured kala-azar patients had a higher IgG2 response (mean OD, 1.5 ± 0.5) than uninduced PBMCs of the same kala-azar patients (mean OD, 0.26 ± 0.06). The difference was statistically significant (P < 0.0001). However, the IgG1 response was not significantly different between the in-

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duced and the uninduced PBMCs (mean OD, 0.43 ± 0.03 versus 0.39 ± 0.04; P = 0.3) (Fig. 3).

**Evaluation of the DNA vaccine construct in BALB/c mice.** The DNA vaccine candidate, expressing the *L. donovani* kinesin motor protein, was prepared by cloning in a pVAX-1 vector. The in vitro translation system confirmed the expression of the kinesin motor domain from the DNA vaccine construct (Fig. 4). Following generation of the vaccine candidate, BALB/c mice were immunized and were finally bled 2 weeks after the final injections. Splenocytes were isolated from both control and test groups of mice, and proliferation assays were carried out, followed by cytokine profile analysis using an ELISA. Statistically insignificant (P = 0.09) proliferative responses were observed against expressed protein rLvacc in the splenocytes from mice injected with empty plasmid (0.1275 ± 0.006). However, mice in the group injected with the kinesin motor domain DNA vaccine construct generated a strong proliferative response against rLvacc (0.4060 ± 0.013). The difference was statistically significant (P < 0.0001). The mouse group injected with kinesin immunodominant repeat domain constructs also showed a good proliferative response against rKE16. Concanavalin A controls showed a good proliferative response in all vaccinated groups and the empty plasmid group (Fig. 5).

**Levels of IFN-γ, IL-2, and IL-4 in response to rLvacc.** The supernatants of rLvacc-stimulated splenocytes from control and vaccinated mice were assayed for IFN-γ, IL-2, and IL-4 concentrations. The rLvacc-stimulated splenocytes showed low IFN-γ, IL-2, and IL-4 levels (200.8 ± 12.14, 118.0 ± 3.95, and 63.83 ± 7.12 pg/ml, respectively) in controls injected with empty plasmid, whereas the vaccinated mice showed significantly (P < 0.0001) higher levels of IFN-γ and IL-2 (1,695 ± 1,824 pg/ml) and IL-4 (2,545 ± 338 pg/ml).

![Western blot analysis of rLvacc (35-kDa kinesin motor domain) in kala-azar patients. Lane M, prestained molecular mass marker; lane 1, rLacc probed with VL-positive patient serum; lane 2, rLacc probed with VL-positive patient serum; lane 3, rLvacc probed with VL-positive patient serum; lane M1, prestained molecular mass marker; lanes 2 and 3, rLacc probed with VL-negative patient serum.](image1)

![Lymphoproliferative responses to rLvacc in cured and healthy kala-azar patients. The proliferative response in cured VL patients (mean OD, 0.4628 ± 0.014) was compared to nonendemic healthy controls (mean OD, 0.1046 ± 0.003).](image2)

![IgG2 and IgG1 production in response to rLvacc stimulation in cured kala-azar patients. rLvacc-stimulated IgG2 production (mean OD, 1.5 ± 0.5) was compared to production in unstimulated PBMCs of the same kala-azar patients (mean OD, 0.26 ± 0.06). The IgG1 production in the induced (mean OD, 0.43 ± 0.03) and the uninduced PBMCs (mean OD, 0.39 ± 0.04) is also shown (P < 0.3).](image3)
52.52 and 413.3 ± 12.89 pg/ml, respectively) but lower levels of IL-4 (57.67 ± 3.69 pg/ml). The difference in IL-4 levels between the vaccinated and the empty plasmid group was statistically insignificant (P = 0.4587) (Fig. 6). The mice injected with the kinesin immunodominant construct showed a mixed cytokines profile. The levels of IFN-γ, IL-2, and IL-4 were 419.2 ± 41.48, 179.2 ± 12.94, and 285.5 ± 12.44 pg/ml, respectively (Fig. 6).

**Immune response to whole parasite.** Stimulation with whole-cell lysate resulted in a Th1-specific cytokine profile. This mouse group showed significantly (P < 0.0001) higher levels of IFN-γ and IL-2 (1,134 ± 92.40 and 406.7 ± 30.29 pg/ml) but lower levels of IL-4 (60.50 ± 2.51 pg/ml) (Fig. 7).

**DISCUSSION**

T lymphocytes participate in the immune response to *L. donovani* infection by producing different cytokines of Th1 and Th2 types. Th1 and Th2 cells can be distinguished by the cytokines they secrete: Th1 cells secrete activators of cell-mediated immunity, such as IFN-γ, while Th2 cells secrete cytokines, such as IL-4, which promote antibody responses. Infection by *L. donovani* in humans induces T-cell anergy as assessed by the depression of delayed-type hypersensitivity reactions and failure of peripheral blood T cells to proliferate (15, 17) and to produce IFN-γ and IL-2 in response to *Leishmania* antigens (4, 7). Cytokine analysis reveals enhanced induction of IFN-γ, IL-10, and/or IL-4 mRNA in tissues (12, 19) and the enhanced presence of IL-4 in the circulation (31) of visceral leishmaniasis patients. While the presence of these cytokines suggests a coexistence of Th1- and Th2-like responses in the clinical stage of the disease, the absence of IL-2 points to the dominance of the Th2 response. Recovery from visceral leishmaniasis is always associated with immunity to subsequent infection and induction of Th1 cytokines which activate macrophages to kill the intracellular organisms, primarily through a nitric oxide-mediated mechanism (23). Cured individuals demonstrate an increase in the number of IFN-γ, IL-2 neutrophils, eosinophils, and NK cells, besides an increased in the frequency of IL-2+ monocytes. Moreover, in cured individuals, on reexposure to leishmania antigens, T cells rapidly expand and activate host protective effector mechanisms, predominantly a Th1-type immune response (21). Therefore, the antigens that are involved in the induction and recall of such memory Th1 cells are considered to be of great interest in the vaccine design strategy against visceral leishmaniasis.

In the present study we have selected the kinesin motor domain for its possible immunogenic activity against visceral leishmaniasis infection. The kinesin gene had already been characterized from various visceralizing species of *Leishmania*, and the immunodominant repeat domain of the kinesin gene showed its importance in diagnosing sera from human kala-
azar patients (3, 28). Although this antigen has been used successfully for serodiagnosis of active kala-azar cases, it is difficult to assign any protective function for the specific antibodies at such high titers produced against this and other similar antigens (6). So, we carried out further analysis of the whole kinesin gene using MHC prediction software, as mentioned earlier, which reveals clusters of MHC-I and -II binding epitopes in the kinesin motor domain region. On the basis of the above findings, we studied lymphoproliferative and antibody responses to the kinesin motor domain in PBMCs from cured kala-azar patients. The present study has shown favorable results and a high potential of our rLvacc as a future vaccine candidate. This is evident from the good proliferation of PBMCs from cured kala-azar patients at a concentration of 5 μg/well of rLvacc (Fig. 3). We also observed high IgG2 titers in culture supernatants of rLvacc-induced PBMCs from cured kala-azar patients. It has been reported that IgG2 production is stimulated by the IFN-γ and IL-12 cytokines (11), whereas IgG1 production is downregulated by IFN-γ (20). Therefore, our findings suggest that Leishmania donovani-primed PBMCs from cured kala-azar patients after stimulation with rLvacc exhibit a strong Th1-type response, which is protective in nature. Reports of observed patterns of parasite-specific immune responses indicate that IFN-γ plays an important role in protection against visceral leishmaniasis (10). As expected, rLvacc did not induce proliferation or antibody production in healthy subjects, suggesting its specificity toward L. donovani infection.

On the basis of these preliminary findings, we then generated a DNA vaccine construct expressing the kinesin motor domain for immunization in BALB/c mice. It is well accepted now that DNA vaccines induce strong cellular immune response against various intracellular parasites and bacterial species compared to peptide or other conventional vaccines (1). In this approach, peptide synthesized under the direction of the plasmid DNA is brought to the surface of cells and displayed by MHC class I molecules recognized by CD8+ cytotoxic T cells (26). In the present study, a DNA vaccine construct expressing the kinesin motor domain of L. donovani induced strong T-cell proliferation in vaccinated mouse groups, and the IFN-γ and IL-2 production levels mediated by this vaccine construct were significantly higher than that observed in mice injected with empty plasmid. The vaccine construct did not induce IFN-γ production in empty plasmid. Moreover, stimulation with whole-cell lysate resulted in a Th1-specific cytokine profile. The difference in IL-4 production between these groups is statistically insignificant, suggesting the inclination of the vaccine candidate toward a Th1 response. We also studied the immunogenic response to the kinesin immunodominant repeat domain in a BALB/c mouse model. The study revealed that the immunodominant domain, which is predominantly a B-cell epitopic region, induces a mixed Th1 and Th2 cytokine phenotype. Although vaccination with the kinesin immuno-

FIG. 6. Cytokine response to rLvacc and rKE16 stimulation in splenocytes from kinesin motor domain-vaccinated and kinesin immunodominant repeat domain-vaccinated mice. The rLvacc-stimulated splenocytes showed low IFN-γ, IL-2, and IL-4 levels (200.8 ± 12.14, 118.0 ± 3.95, and 63.83 ± 7.12 pg/ml, respectively) in the control empty plasmid group, whereas the kinesin motor domain-vaccinated mice had higher levels of IFN-γ and IL-2 (1.695 ± 52.52 and 413.3 ± 12.89 pg/ml, respectively) but lower levels of IL-4 (57.67 ± 3.69 pg/ml). The splenocytes from the kinesin immunodominant-vaccinated group showed a mixed profile with IFN-γ, IL-2, and IL-4 levels of 419.2 ± 41.48, 179.2 ± 12.94, and 285.5 ± 12.44 pg/ml, respectively.

FIG. 7. Cytokine response to whole-cell lysate of L. donovani (KE-16) in splenocytes from the kinesin motor domain-vaccinated group. The mice had higher levels of IFN-γ and IL-2 (1,134 ± 92.40 and 406.7 ± 30.29 pg/ml) but lower levels of IL-4 (60.50 ± 2.51 pg/ml).
dominant repeat domain leads to IFN-γ production by splenocytes, coproduction of IL-4 may inhibit IFN-γ and macrophage activation. Moreover, it may also lead to B-lymphocyte activation, cosinophil production, and IgG1 and IgE synthesis, thus deviating from the intracellular parasite immunity toward humoral response. This type of study leads to the first step of antigen selection in experimental models and in human beings, in order to evaluate the ability to induce protection against Leishmania infection.

Taken together, our studies suggest that rLvacc expressed from the kinesin motor domain region of \( L. \) \( donovani \) (strain KE-16) induces a strong Th1-type immune response important for protection against visceral leishmaniasis. These findings pave the way to test further that the kinesin motor domain is a potential vaccine candidate. Furthermore, studies to assess the immunoprotective role of rLvacc in a mouse model are under way.

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