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Immunization with the DNA-Encoding N-Terminal Domain of Proteophosphoglycan of Leishmania donovani Generates Th1-Type Immunoprotective Response against Experimental Visceral Leishmaniasis

Mukesh Samant,* Reema Gupta,* Shraddha Kumari,* Pragya Misra,* Prashant Khare,* Pramod Kumar Kushawaha,* Amogh Anant Sahasrabuddhe,† and Anuradha Dube2*

Leishmania produce several types of mucin-like glycoproteins called proteophosphoglycans (PPGs) which exist as secretory as well as surface-bound forms in both promastigotes and amastigotes. The structure and function of PPGs have been reported to be species and stage specific as in the case of Leishmania major and Leishmania mexicana; there has been no such information available for Leishmania donovani. We have recently demonstrated that PPG is differentially expressed in sodium stiboglucone-sensitive and -resistant clinical isolates of L. donovani. To further elucidate the structure and function of the ppg gene of L. donovani, a partial sequence of its N-terminal domain of 1.6 kb containing the majority of antigenic determinants, was successfully cloned and expressed in prokaryotic as well as mammalian cells. We further evaluated the DNA-encoding N-terminal domain of the ppg gene as a vaccine in golden hamsters (Mesocricetus auratus) against the L. donovani challenge. The prophylactic efficacy to the tune of ∼80% was observed in vaccinated hamsters and all of them could survive beyond 6 mo after challenge. The efficacy was supported by a surge in inducible NO synthase, IFN-γ, TNF-α, and IL-12 mRNA levels along with extreme down-regulation of TGF-β, IL-4, and IL-10. A rise in the level of Leishmania-specific IgG2 was also observed which was indicative of enhanced cellular immune response. The results suggest the utility of L. donovani ppg as a potential DNA vaccine against visceral leishmaniasis. The Journal of Immunology, 2009, 183: 470–479.

Leishmania produce a range of glycoconjugates containing phosphoglycan (PG)7 that includes membrane-bound lipophosphoglycan and proteophosphoglycans (PPG), as well as secreted PG and acid phosphatase (1). These glycoconjugates have been shown to play important roles in parasite virulence both in vector and the mammalian host (2–6). Among these PPGs are newly described mucin-like glycoproteins present on the surface and secreted by both promastigote and amastigote stages of Leishmania. These proteins are thought to be important in the transmission, invasion, and subsequent intracellular survival of parasites (7). The structure and function of PPGs are species and stage specific as in the case of Leishmania major and Leishmania mexicana, but no such information has hitherto been available for Leishmania donovani (8–11). We have very recently demonstrated the expression of PPG to be higher in sodium stiboglucone-resistant clinical isolates of L. donovani as compared with the sensitive ones (11).

It was proposed earlier that the assembly of PGs in the parasites may provide targets for the rational development of new Leishmania-specific drugs/vaccines (12). Considerable efforts were made to elucidate the biosynthetic pathway of the lipid-linked PGs in lipophosphoglycans (2, 13–16). Much less is known about the biosynthesis of the PPGs since a prerequisite for such studies is sequence information about their protein backbones. To date, the genes encoding PPG from L. major and L. mexicana have only been cloned. Ilg et al. (17) identified the ppg1 gene of L. major promastigotes encoding a membrane-bound PPG consisting of a large central domain of ∼100 repeats of the sequence APSASSS-SA(P/S)SSSSS(9/S). The N-terminal to the central repetitive domain is a nonrepetitive sequence containing a leucine-rich repeat motif (17, 18) and the carboxyl terminus consists of a second nonrepetitive region terminating in a hydrophobic amino acid sequence compatible with GPI addition (9, 17). This gene is distinct from the single copy ppg2 gene of L. mexicana that contains a different serine-rich repetitive sequence (9) and encodes a secreted nonfilamentous PPG that is expressed by both promastigotes and amastigotes. The sequence of another ppg gene, i.e., ppg3 in L. major, revealed that this 4.3-kb long gene (19), containing N-terminal domain and central serine-rich repetitive sequence, is different from ppg1 and ppg2 genes of L. major and L. mexicana, respectively, and comparatively more simpler in amino acid composition. Moreover, it has extracellular localization and showed its homology to other surface Ag proteins as observed by Blastp/TBlastN/BLASTX analysis. In the present communication, the
characterization of the **ppg** gene of *L. donovani* was initiated with the N-terminal domain since it contains the majority of antigenic determinants (20) (Fig. 1).

Active visceral leishmaniasis (VL) is associated with the absence of parasite-specific cell-mediated immune response (21, 22). It has been suggested that disease susceptibility in VL is due to the lack of Th1 response rather than the presence of Th2 response (23). In the clinical model *Leishmania*, Ag-specific expansion of both Th1 and Th2 subsets capable of producing IFN-γ as well as IL-4 and IL-10 are found in cured VL patients (24, 25). Since the current scenario for control measures rely only on chemotherapy and the available chemotherapeutic agents being inadequate, expensive, and often toxic (26, 27), an alternative choice is immunophylaxis. Keeping this in view, we further formulated an effective DNA-encoding N-terminal domain of the **ppg** gene and evaluated it as a vaccine candidate against challenge of *L. donovani* in golden hamsters, a good model for VL as it develops a progressive, lethal disease which very closely mimics the disease in humans and as such has been used for vaccination studies (28, 29). The studies were therefore performed to assess whether the resultant protective ability of genetically immunized hamsters was due to a polarized Th1-like response, or to a mixed Th1/Th2-like response, representative of a clinically cured VL scenario. Our studies indicate a strong protective response in VL depending on T cell functional-Th1-type cytokine response detected for the first time through real-time quantitative RT-PCR and induction of leishmanicidal effector molecules.

**Materials and Methods**

**Cell culture**

Baby hamster kidney cell line BHK-21 was procured from the National Centre for Cell Science (Pune, India). Cells were grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM NaHCO₃, 20 mM HEPES, along with 10% FCS (Life Technologies at 37°C in the presence of a 5% CO₂ supply.

**Animals**

Laboratory inbred male golden hamsters (*Mesocricetus auratus*, 45–50 g) from the Institute’s animal house facility were used for experimental purposes with prior approval of the animal ethics committee of the Central Drug Research Institute (Lucknow, India). They were housed in climatically controlled rooms and fed with standard rodent food pellet (Lipton India) and water ad libitum.

**Parasites**

The *L. donovani* strains sodium stibogluconate-resistant 2039 (procured from a patient admitted to the Kala Azar Medical Research Centre, Muzaffarpur, Bihar) and WHO reference strain Dd8 were cultured in vitro as described elsewhere (30). The strains have also been maintained in hamsters through serial passage, i.e., from amastigote to amastigote. For bulk cultivation, promastigotes were grown in L-15 medium (Sigma-Aldrich) with L-glutamine, supplemented with 10% tryptose phosphate broth (Hi-media), 0.1% gentamicin, and 10% FBS (Life Technologies). Parasites were harvested after 3–4 days of culture (31).

**Cloning and expression of N-terminal domain of **ppg** (1.6 kb)**

*L. donovani* strain 2039 genomic DNA was isolated from 10⁸ promastigotes, washed, and suspended in NET buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA) and incubated with proteinase K (1 mg/ml; Invitrogen Life Technologies) and 0.5% SDS at 50°C for 4 h. Nucleic acids were extracted by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Genomic DNA was spooled and subjected to RNase (100 μg/ml) treatment. N-terminal domain of the **ppg** gene was amplified using Taq Polymerase (Sigma-Aldrich) lacking a 3’–5’ exonuclease activity. PCR was performed using **ppg**-specific primers (based on the *L. major* **ppg3** gene sequence): forward, 5’-GGATCCACCATGTCTTTTCATAGGGCGCCGG-3’ and reverse, 5’-GGATCCATCTGGAATTGTGAGGTGCACCAATCG-3’ (*BamHI* site underlined) in a Thermocycler (Bio-Rad) under conditions at one cycle of 94°C for 4 min, 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min 30 s, and finally one cycle of 72°C for 10 min. Amplified PCR product was electrophoresed in agarose gel and eluted from the gel by GenElute Columns (Qiagen). Eluted product was

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**FIGURE 1.** The schematic structure of PPG (11, 75) (A) and Ag determinants in **ppg** using Ag determination software (B).

![Diagram](http://www.jimmunol.org/)
cloned in pTZ57R/T (T/A) cloning vector (Fermentas) and transformed into competent DH5α cells. The transformants were screened for the presence of recombinant plasmids with the ppg insert by gene-specific PCR under similar conditions as previously mentioned. Isolated positive clones were sequenced from Delhi University (New Delhi) and submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nuccore/119656394; accession no. EF141073). ppg was further subcloned at the BamHI site in bacterial pET28a (Novagen) and mammalian expression pcDNA3 (Invitrogen) vectors.

The expression of PPG was checked in bacterial cells by transforming the ppg + pET28a construct in Escherichia coli Rosetta strain. The transformant cells were inoculated into 5-ml test tube culture medium (Luria-Bertani) and allowed to grow at 37°C in a shaker at 220 rpm. Cultures in logarithmic phase (at OD_{600} of ~0.5–0.6) were induced for 3 h with 1.0 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C. After induction, cells were lysed in 5× sample buffer (0.313 M Tris-HCl (pH 6.8), 50% glycerol, 10% SDS, and 0.05% bromphenol blue, with 100 mM DTT) and whole cell lysates (WCL) were analyzed by 12% SDS-PAGE (32). Uninduced control culture was analyzed in parallel. The separated proteins from the polyacrylamide gel were transferred onto a nitrocellulose membrane in a semidy blot apparatus (Amersham) as described earlier (33). Membrane was incubated for 1 h in blocking buffer followed by 2-h incubation at room temperature with mouse anti-His Ab (Novagen) as primary Ab (1/2500 dilution) and then incubated with goat anti-mouse HRP conjugate Ab (1/10,000; Bangalore Genei) for 1 h at room temperature. The blot was developed using an ECL kit (GE Biosciences).

The expression of PPG was further checked in mammalian cells by transfecting the ppg + pcDNA3 construct in BHK cells. For transfection and selection of the pcDNA3-expressed vector DNA was isolated using an Endofree plasmid purification Maxi kit as per the manufacturer’s protocol (Qiagen). For confirmation and cellular localization of the proteins expressed by the ppg + pcDNA3 construct, 2 × 10^5 BHK cells were grown in four-chamber slides and transfected with different sets of plasmids, the blank pcDNA3 plasmid (as negative control), ppg + pcDNA3 construct, and gfp + pcDNA3 construct (as positive control) and cotransfected with ppg + gfp-containing plasmids in serum-free DMEM (Life Technologies) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Expression was confirmed by RT-PCR and fluorescence microscopy. For RT-PCR, RNA was isolated from the transfected BHK cell line using TRIzol (Sigma-Aldrich) and the cDNA was synthesized using M-MLV reverse transcriptase as per the manufacturer’s instructions (Promega). cDNA samples were used as a template to amplify the ppg gene using gene-specific primers. The expression of ppg at the appropriate cellular localization was also confirmed by immunofluorescence using polyclonal Ab raised in rabbit against deglycosylated and dephosphorylated native filamentous PPG of *L. major* (gift from Dr. E. Handman, The Walter and Eliza Hall Institute of Medical Sciences, Melbourne, Australia). BHK cells were plated in four-chamber slides and allowed to grow for 24 h. For detection of intracellular PPG, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 10 min followed by incubation with the same primary anti-serum and secondary anti-rabbit FITC conjugate (Amersham) and pcDNA3 plasmid as template. The amplified product was resolved in 1% agarose gel, transferred onto nylon membranes (Hybond-N+; Amersham Biosciences), and subjected to Southern blot analysis with a ppg-specific probe (34, 35).

**Measurement of PPG expression in mammalian cells**

For detection of the ppg gene level in hamsters, total DNA was extracted from the muscle of ppg + pcDNA3- immunized as well as pcDNA3 blank vector-immunized hamsters using a NucleoSpin nucleic acid purification kit (MACHERY-NAGEL) as per the manufacturer’s protocol. PCR amplification of the ppg gene was conducted using muscle DNA of hamsters as template as described earlier (36). For probe synthesis, PCR was performed using a mix of forward and reverse 5′-end labeled UTPs (Roche) and ppg + pcDNA3 plasmid as template. The amplified product was resolved in 1% agarose gel, transferred onto nylon membranes (Hybond-N+; Amersham Biosciences), and subjected to Southern blot analysis with a ppg-specific probe (34, 35).

**Preparation of soluble L. donovani (SLD) promastigote Ag**

SLD promastigote Ag was prepared according to the method described by Scott et al. (36) and modified by Choudhry et al. (37). Briefly, late phase promastigotes (10^7) were harvested from 3- to 4-day cultures, washed four times in cold PBS, resuspended in PBS containing protease inhibitors mixture (Sigma-Aldrich), and sonicated (Soniprep-150) for two periods of 1.5 min each in ice, (separated by an interval of 3 min) at medium amplitude. The sonicated sample was subjected to rapid freeze-thawing four times using liquid nitrogen and left at 4°C for 1 h for complete extraction of soluble Ag. The suspension was centrifuged at 4,000 × g for 20 min at 4°C followed by ultracentrifugation at 40,000 × g for 30 min. After assessing protein contents, the Ag was aliquoted and stored at −70°C.

**Measurement of delayed-type hypersensitivity (DTH) in hamsters**

DTH was performed by injecting 50 μg/50 μl of SLD in PBS intradermally into one footpad and PBS alone into the other one of each of the vaccinated and unvaccinated controls. The response was evaluated 48 h later by measuring the difference in footpad swelling between the two with and without SLD for each animal (38).

**Assessment of parasitic burden in vaccinated hamsters**

The prophylactic efficacy of all of the experimental groups was assessed on necropsy at different time intervals, i.e., on days 0, 45, 60, 120, and 180 p.c. The impression smears/touch blots of different organs, namely, spleen, liver, lung, bone marrow (femur bone) of experimental animals were made and the criteria for the assessment of parasitic burden was based on the counting of the number of amastigotes/1000 cell nuclei in each organ. The percentage of inhibition (PI) of parasite multiplication was calculated in comparison to the unvaccinated control using the following formula: PI = No. of parasites from infected control − no. of parasites from the vaccinated group/no. of parasite count from infected control × 100.

**Immunological assays**

For evaluation of cellular and Ab responses, peritoneal exudate cells, inguinal lymph nodes, and blood were collected from hamsters on necropsy at various time intervals, i.e., day 0, 45, 60, 120, and 180 p.c. The impression smears/touch blots of different organs, namely, spleen, liver, and bone marrow (femur bone) of experimental animals were made and the criteria for the assessment of parasitic burden was based on the counting of the number of amastigotes/1000 cell nuclei in each organ. The percentage of inhibition (PI) of parasite multiplication was calculated in comparison to the unvaccinated control using the following formula: PI = No. of parasite count from infected control − no. of parasites from the vaccinated group/no. of parasite count from infected control × 100.

**Assessment of lymphoproliferative responses in vaccinated hamsters**

Lymph nodes of hamsters were excised aseptically and processed for the isolation of lymphocytes (39). The lymphocytes were suspended to 10^6/ml and cultured at 10^6 cells/well in 96-well flat-bottom tissue culture plates (Nunc). One hundred microliters of Con A (10 μg/ml; Sigma-Aldrich) or SLD (10 μg/ml) was added to each well in triplicate. Wells without stimulants served as negative controls. Cultures were incubated at 37°C in a CO_2 incubator for 3 days in the case of mitogens and for 5 days in the case of SLD Ags. Eighteen hours before termination of culture, 0.5 μg/ml PHA (Hyclone) was added to each well. On days 0, 45, 60, 120, and 180 postchallenge (p.c.), three to five hamsters per group were necropsied for parasitological and immunological assessment of progression of VL.
was calculated as mean cpm of stimulated culture/mean cpm of unstimulated control. SI values of >2.5 were considered as positive response.

Assessment of level of NO activity in macrophages of vaccinated hamsters

The presence of nitrite (NO₂⁻) content was assessed using Griess reagent in the culture supernatants of naive hamster peritoneal macrophages after the exposure with supernatant of stimulated lymphocyte cultures from all of the study groups. The supernatants (100 µl) collected from macrophage cultures 24 h after incubation were mixed with an equal volume of Griess reagent (Sigma-Aldrich) and left for 10 min at room temperature. The absorbance of the reaction was measured at 540 nm in an ELISA reader (40).

Estimation of expression of mRNA cytokines by real-time PCR

Real-time PCR was performed to assess the expression of mRNAs for various cytokines and inducible NO synthase (iNOS) in splenic cells. Splenic tissues were taken from each of the three individual animals randomly chosen from different groups. Total RNA was isolated using Tri-reagent (Sigma-Aldrich) at different time intervals and quantified by using Gene-quant (Bio-Rad). One microgram of total RNA was used for the synthesis of cDNA using a first-strand cDNA synthesis kit (Fermentas). PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 40 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s. Real-time quantitative PCR was conducted as per the protocol described earlier (43). Briefly, it was carried out with 12.5 µl of SYBR green PCR master mix (Bio-Rad), 1 µg of cDNA, and primers at a final concentration of 300 nM in a final volume of 25 µl. PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 40 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s per cycle using the iQ5 multicolor real-time PCR system (Bio-Rad). cDNAs from infected hamsters were used as “comparator samples” for quantification of those corresponding to test samples. All quantifications were normalized to the housekeeping gene HGPRT. A no-template control (cDNA) was included to eliminate contaminations or nonspecific reactions. The cycle threshold (Ct) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value.

Table I. Sequence of forward and reverse primers used for quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>HGPRT forward</td>
<td>5'-GATAGATCCACAATCCGATATCC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TACCTCTCAAACCTGACATTCC-3'</td>
</tr>
<tr>
<td>TNF-α forward</td>
<td>5'-TCCTCCTCCCTGCTCTTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GTGTTGATGCTGTTAGG-3'</td>
</tr>
<tr>
<td>IFN-γ forward</td>
<td>5'-GCTGCCTTGGAGGAGATTGAG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTTTCCTTATAGATCCAGT-3'</td>
</tr>
<tr>
<td>IL-12 forward</td>
<td>5'-TTGTCGCAAGGATTGATTTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TGGTTGATGCTGTTAGG-3'</td>
</tr>
<tr>
<td>TGF-β forward</td>
<td>5'-ACGGAGAAAGACTGGTTGTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCTGCTGTTGAAGAAGTTAG-3'</td>
</tr>
<tr>
<td>IL-4 forward</td>
<td>5'-GCCATTCCCTCCGCTCTCCTCC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCCATTCCCTCCGCTCTCCTCC-3'</td>
</tr>
<tr>
<td>IL-10 forward</td>
<td>5'-TGGCACAACCTTAAGCAGAAATG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CGAGCGACACCACATGAG-3'</td>
</tr>
<tr>
<td>iNOS forward</td>
<td>5'-AGGATCACAGGCGACACATC-3'</td>
</tr>
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</table>

FIGURE 2. Cloning and expression of ppg. Clone confirmation of ppg in pET28a vector. M, 1-kb molecular mass marker; lane I, BamHI-digested ppp-ppg/pET28a plasmid construct; lane 2, XhoI-digested plasmid; lane 3, undigested plasmid (I). Expression of rPPG in prokaryotic cells. WCL of transformed E. coli separated on 12% acrylamide gel and stained with Coomassie blue. II, A, Western blot analysis using anti-His mAb. M, Molecular mass markers; Lane 1, WCL before IPTG induction; lane 2, WCL after IPTG (1.0 mM) induction at 37°C (II, B). Clone confirmation of ppg in mammalian expression pcDNA3 vector. M, 1-kb molecular mass marker; lane I, BamHI-digested ppg-PCDNA3 plasmid construct; lane 2, NotI-digested plasmid; lane 3, XhoI-digested plasmid; lane 4, undigested plasmid (III, A). In vitro expression of ppg in mammalian BHK cell line by RT-PCR (III, B) and fluorescence microscopy (IV). Lanes 1–4 (III, A) and A–D (IV) are pcDNA3 transfected; gfp-ppg-PCDNA3 transfected; and ppg-PCDNA3 transfected and ppg-ppg cotransfected BHK cells, respectively. In vivo expression of ppg in the hamster, PCR amplification of genomic DNA isolated from muscle tissue of hamsters 3 wk after immunization with pcDNA3 and ppg-ppg-PCDNA3 plasmid using ppg-specific primers, M, 1-kb molecular mass marker. Lane I, pcDNA3; lane 2, ppg-PCDNA3 (V, A). Southern blot analysis of PCR-amplified ppg from the genomic DNA isolated from the muscle tissue of the hamster using the ppg probe (V, B).

Determination of antileishmanial Ab responses in hamsters

The levels of antileishmanial Abs-IgG and its isotypes, IgG1 and IgG2, in sera samples from hamsters of different groups were measured as described earlier (41). The 96-well ELISA plates (Nunc) were coated with SLD (0.2 µg/100 µl/well) overnight at 4°C and blocked with 1% BSA at room temperature for 1 h. The optimum dilution of sera was standardized at 1/200 for IgG, IgG1, and IgG2 for 2 h at room temperature. HRP-conjugated goat anti-hamster IgG (H + L) (Serotec) and biotin-conjugated mouse anti-Armenian and anti-Syrian hamster IgG1 (for IgG1) (BD Pharmingen) as well as mouse anti-Syrian hamster IgG2 (BD Pharmingen) were added for 1 h at room temperature at 1/800 dilutions. IgG1 and IgG2 plates were further incubated with streptavidin-conjugated peroxidase (Sigma-Aldrich) for 1 h. Finally, the substrate O-phenylenediamine dihydrochloride (Sigma-Aldrich) was added and the plate was read at 492 nm.

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Differences in gene expression were calculated by the comparative Ct method (43). This method compares test samples to a comparator sample and uses results obtained with a uniformly expressed control gene (HGPRT) to correct for differences in the amounts of RNA present in the two samples being compared to generate a Ct value. Results are expressed as the degrees of difference between Ct values of test and comparator samples.

Postchallenge survival
Survival of hamsters belonging to group 4 was checked until day 180 p.c. in comparison to the normal hamsters (group 1). Animals in all of the groups were given proper care and were observed for their physical conditions until their survival period. Survivals of individual hamsters were recorded and mean survival period was calculated.

Statistical analysis
Results are expressed as mean ± SD of three to five individual animals per group at designated time points. Three replicates were done. The results (pooled data of three experiments) were analyzed by one-way ANOVA followed by Dunnet’s or Tukey’s post test where appropriate. All of the analyses were done using GraphPad Prism (version 3.03) software.

Results
PPG was cloned, sequenced, and expressed in vitro in E. coli Rosetta strain and in BHK cells as well as in vivo in hamsters
The ppg gene of L. donovani was successfully cloned and sequenced in the T/A cloning vector, which was 95% homologous with L. major ppg3. It was further cloned in right orientation under the T7 promoter in bacterial expression pET28a (Fig. 2f) as well as mammalian expression vector pcDNA3 (Fig. 2III, A). The expression status of the cloned gene when checked in vitro at the protein level in the E. coli Rosetta strain by Western blotting using anti-His Ab exhibited the predicted ~65.120-kDa recombinant protein (Fig. 2II, B). In BHK cells, the expression level was established at the RNA level by RT-PCR depicting a 1.6-kb band in ppg + gfp cotransfected and ppg transfected but not in vector as well as gfp-transfected cells (Fig. 2III, B). The expression at the protein level was confirmed by fluorescence microscopy using Ab raised in rabbits against deglycosylated and dephosphorylated native filamentous PPG of L. major. The BHK cells transfected with gfp + pcDNA3, ppg + pcDNA3, as well as gfp- and ppg-containing plasmids exhibited bright green fluorescence, indicating the expression of PPG inside the cells. Blank vector-transfected BHK cells showed no fluorescence (Fig. 2IV).

The expression level of PPG was also confirmed in vivo at the DNA level by PCR and Southern blotting from immunized hamster tissues. The ppg signal was clearly visible in tissue from hamsters injected with ppg + pcDNA3 construct but not in those hamsters injected with blank vector (Fig. 2V, A). To show the sensitivity and specificity of PCR, the PCR products were detected by Southern blot hybridization using ppg sequence-specific probe. As shown in Fig. 2V, B, hamsters immunized with the ppg + pcDNA3 construct showed a significant amount of ppg DNA in muscle tissue.

Vaccination with ppg DNA-induced optimum protection against L. donovani challenges
The ppg DNA-vaccinated hamsters were found to be protected from the challenge infection of L. donovani, as indicated by their weight gain with time like normal animals. In contrast, there was significant weight loss (p < 0.001) in any of the animals of the

![FIGURE 3.](http://www.jimmunol.org/)

Body weight (A), spleen weight (B), and liver weight (C) in g as well as parasite burden (no. of amastigotes per 1000 cell nuclei) in the spleen (D), liver (E), and bone marrow (F) on days 0, 45, 90, 120, and 180 p.c. Significance values indicate the difference between the vaccinated groups and infected group (*, p < 0.05; **, p < 0.01; and ***, p < 0.001).
infected and vector control groups (Fig. 3A). There was an absence of hepatosplenomegaly in the vaccinated group that is normally associated with the challenge infection (Fig. 3, B and C). A positive correlation of parasite loads with splenomegaly and hepatomegaly was observed among the experimental and control groups. An increase from $10^3$ to $10^4$ parasites in all of the groups, except in the ppg DNA-vaccinated group, was seen in Giemsa-stained splenic smears from days 45 to 120 p.c. (Fig. 3D). In the vaccinated group, parasite loads decreased from $2 \times 10^2$ on day 45 to a negligible level ($<10$ subsequently) ($p < 0.001$) by day 180 p.c., rendering them difficult to discern by microscopy (data not shown). Similarly, in liver and bone marrow, parasite loads decreased sharply after day 45 p.c. and parasites were essentially absent by day 180 p.c. in the same vaccinated group (Fig. 3, E and F). Cultivation of the spleen, liver, and lymph node tissues from the vaccinated hamsters in vitro yielded no promastigotes after prolonged incubation for 3 wk.

The ppg DNA-vaccinated hamsters survived the challenges of *L. donovani* and remained healthy until the day of termination of the experiment, i.e., 6 mo p.c. In contrast, hamsters vaccinated with the pcDNA3 vector and infected control survived for only 2–3 mo.

**ppg DNA vaccination alters *Leishmania*-specific IgG and its isotypes**

The serum levels of leishmanial Ag-specific IgG and its isotypes (IgG1 and IgG2) from all of the groups were assessed by ELISA. The anti-*Leishmania* IgG and IgG1 were elevated progressively with time to a high level in all groups, except the ppg DNA-vaccinated, in which case they remained essentially the background levels of the nonimmunized and unchallenged normal and blank vector immunized (Fig. 4, D and E). In contrast, ppg DNA-vaccinated animals were the only group (Fig. 3F) that showed a significant elevation by 1- to 2-fold over the others ($p < 0.01$) in the level of IgG2. As a measure of CMI, the elevation of IgG2 was consistent with the development of effective immune responses.

### FIGURE 4.

DTH response (mm) (A), LTT response (SI value) to SLD (B) and Con A (C), and *Leishmania*-specific IgG (D) and its isotypes IgG1 (E) and IgG2 (F) in ppg DNA-vaccinated hamsters in comparison to the unimmunized infected controls, vector-immunized controls, and uninfected normal hamsters on days 0, 45, 60, 120, and 180 p.c. Significance values indicate the difference between the vaccinated groups and infected group (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).
ppg DNA immunization induces NO production in vaccinated hamsters

Lymphocyte-mediated activation of macrophages to produce NO for leishmanicidal activities was found to differ between control and experimental groups of hamsters. Supernatants from stimulated lymphocytes of hamsters vaccinated with the ppg DNA when incubated with naive macrophages produced significant (p < 0.001) amounts of nitrite (19.5 ± 1.94) which was ~4-fold more than that of unvaccinated infected controls and ~2.3-fold more than the vector control group on day 45 p.c. Furthermore, the level of nitrite was increased incredibly on days 60 (21.5 ± 2.63), 120, and 180 p.c. (24.13 ± 3.17). Comparatively, a very low amount of nitrite was produced by unvaccinated (5.4 ± 1.3) and vector (5.827 ± 0.6378) control groups on day 45 p.c. (Fig. 5A). Similarly, LPS (100 μg/ml, used as positive control)-stimulated cells from the vaccinated and normal control groups demonstrated good and significant (p < 0.001) nitrite production until day 180 p.c. while infected control and vaccinated with vector alone groups showed less nitrite production until they survived (Fig. 5B).

ppg DNA vaccination generates Th1-type cytokine profile as determined by quantitative real-time PCR

Impairment of the CMI response during active VL is reflected by marked T cell anergy-specific to Leishmania Ags (44, 45). Since optimum protective efficacy was observed in ppg DNA-vaccinated hamsters, the expression of Th1 and Th2 mRNA cytokines was further evaluated by real-time PCR on days 45 and 60 p.c. The expression of iNOS transcripts was observed to be significantly (p < 0.01 and p < 0.05, respectively, on days 45 and 60) elevated in ppg DNA-immunized hamsters (Fig. 6) at both time points of the study. Similarly, the expression of TNF-α was also significantly higher on day 45 (p < 0.001) and day 60 (p < 0.05) in the vaccinated group (ΔCt = 1.5 ± 0.4 and 2.3 ± 0.3, respectively) in comparison to the L. donovani-infected group (ΔCt = 7.7 ± 0.8 and 5.6 ± 2.5, respectively, on days 45 and 60). The expression of IFN-γ, although variable at different time points, was suppressed in the infected group (ΔCt = 6 ± 0.8, day 45 p.c.), but was significantly higher in the vaccinated group on day 45 (p < 0.001) and day 60 (p < 0.05) p.c. and was at par with the normal ones. Similar was the case with IL-12 which was least expressed in the infected group on day 45 p.c. but was significantly (p < 0.01) expressed by 3.5- to 4.0-fold in vaccinated hamsters on days 45 and 60 p.c.

FIGURE 5. NO production (μM) to LPS (A) and SLD (B) in the naive macrophages coincubated with supernatants of lymphocytes isolated from ppg DNA-immunized hamsters in comparison to the unimmunized infected controls, vector-immunized controls, and uninfected normal hamsters on days 0, 45, 60, 120, and 180 p.c. Significance values indicate the difference between the vaccinated groups and infected group (**, p < 0.01 and ***, p < 0.001).

FIGURE 6. Splenic iNOS and cytokine mRNA expression profile analysis of normal, infected, and vaccinated hamsters on days 45 and 60 p.c. by quantitative real-time RT-PCR. Significance values indicate the difference between ΔCt values of infected to normal and vaccinated groups (*, p < 0.05; **, p < 0.01; and ***, p < 0.001).
and 60 p.c. On the other hand, the expression of Th2 type cytokines, i.e., TGF-β, IL-4, and IL-10, was significantly up-regulated ($p < 0.05$ to $p < 0.01$) in the infected groups compared with the vaccinated hamsters (Fig. 6).

**Discussion**

PPGs exist in both stages of parasites and have the main function in survival and virulence. The elucidation of their primary structure reveals unique proteins, PG structures, and protein carbohydrate linkages which, together with their proposed function(s), provide attractive targets for the development of vaccines and antiparasite drugs (7). To divulge its possible function, it is essential to have knowledge of the genetic makeup of the PPG. We derived the *L. donovani* ppg gene on the basis of the *L. major* ppg3 gene sequence with $\sim$95% homology to *L. major*. To evolve its function, it was necessary to express ppg in a suitable vector. The PPG was expressed in the *E. coli* Rosetta strain with pET28a vector and in BHK mammalian cells (at the RNA and protein levels) as well as in hamsters (at the DNA level from immunized hamster muscle tissues) using the pcDNA3 vector. The successful delivery and the expression of the cloned N-terminal domain of PPG in the BHK cell line as well as hamsters further authenticates its evaluation as a DNA vaccine candidate.

In the present study, all of the hamsters immunized with *ppg* + pcDNA3 and challenged with the virulent Dd8 strain of *L. donovani* survived the lethal challenge and remained healthy until the termination of the experiment at day 180 p.c., whereas all nonimmunized and blank vector-immunized hamsters succumbed to the lethal *L. donovani* challenge within 3–4 mo p.c. The advantages with the DNA vaccines include their being protective, stable with low cost of production, no need of cold chain for distribution, and flexibility of combining multiple genes in a simple construct. To date, the most-studied Ags for DNA vaccination against VL were those previously assayed as recombinant proteins (46–48). Most of them were tested only as single vaccines (48–53) or as heterologous prime boosts (54) or a vaccinia virus construct. To date, the most-studied Ags for DNA vaccination against VL using the pcDNA3 vector. The successful delivery and the expression of the cloned N-terminal domain of PPG in the BHK cell line as well as hamsters further authenticates its evaluation as a DNA vaccine candidate.

Finally, unlike mice where IL-4 and IL-12 direct IgG subclass switching of IgG1 and IgG2a, respectively, such distinct IgG classes remain obscure in hamsters (38, 74). It is believed that hamster IgG1 and IgG2 correspond to mouse IgG1 and IgG2a/ IgG2b, respectively. It has been well established that IgG and IgG1 Abs increase in titer with the *L. donovani* loads (53). The virtual absence of these Abs is thus consistent with the decreasing parasite loads seen in the vaccinated group. The significant increase in the IgG2 levels only in vaccinated animals is indicative of enhanced CMI.

Addendum: The virtual absence of these Abs is thus consistent with the decreasing parasite loads seen in the vaccinated group. The significant increase in the IgG2 levels only in vaccinated animals is indicative of enhanced CMI.
In a nutshell, this study demonstrated a considerably good prophylactic efficacy of a DNA vaccine encoding ppg against experimental VL since all of the vaccinated hamsters continued to survive beyond 6 mo after challenge. These hamsters were protected by a surge in IFN-γ, TNF-α, and IL-12 levels along with extreme down-regulation of TGF-β, IL-4, and IL-10. The observation was well supported by the rise in the level of *Leishmania*-specific IgG2 which is indicative of enhanced CMI. These results, therefore, strongly suggest that the N-terminal domain of ppg, a surface molecule, has the potential of a DNA vaccine candidate to be used in humans.

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Disclosures

The authors have no financial conflict of interest.

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