Identification of Vaccine Candidates for Experimental Visceral Leishmaniasis by Immunization with Sequential Fractions of a cDNA Expression Library

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Visceral leishmaniasis caused by the intracellular parasite *Leishmania donovani* is a significant public health problem in many regions of the world. Because of its large genome and complex biology, developing a vaccine for this pathogen has proved to be a challenging task and, to date, protective recombinant vaccine candidates have not been identified. To tackle this difficult problem, we adopted a reductionist approach with the intention of identifying cDNA sequences in an *L. donovani* amastigote cDNA library that collectively or singly conferred protection against parasite challenge in a murine model of visceral leishmaniasis. We immunized BALB/c mice with plasmid DNA isolated and pooled from 15 cDNA sublibraries (~2,000 cDNAs/sublibrary). Following systemic challenge with *L. donovani*, mice immunized with 6 of these 15 sublibraries showed a significantly reduced (35- to 1,000-fold) hepatic parasite burden. Because of the complexity and magnitude of the sequential fractionation-immunization-challenge approach, we restricted our attention to the two sublibraries that conferred the greatest *in vivo* protection. From one of these two sublibraries, we identified several groups of cDNAs that afforded protection, including a set of nine novel cDNAs and, surprisingly, a group of five cDNAs that encoded *L. donovani* histone proteins. At each fractionation step, the cDNA sublibraries or the smaller DNA fractions that afforded *in vivo* protection against the parasite also induced *in vitro* parasite-specific T helper 1 immune responses. Our studies demonstrate that immunization with sequential fractions of a cDNA library is a powerful strategy for identifying anti-infective vaccine candidates.

In some regions of the world visceral leishmaniasis (VL), caused by the protozoan parasite *Leishmania donovani*, is a significant clinical and public health problem. Active VL is a progressive, fatal infection characterized by fever, hepatosplenomegaly, cachexia, pancytopenia, and the absence of parasite-specific cell-mediated immune responses (8, 9). Recent epidemics in Sudan and India have resulted in >100,000 deaths. VL has also been increasingly recognized as an opportunistic infection in individuals infected with the human immunodeficiency virus (3).

A vaccine for this severe form of leishmaniasis is not available. Several vaccination strategies against experimental VL have been attempted, however, with limited success. For example, immunization of mice with killed *L. donovani* parasites, crude antigen fractions, and a purified *L. donovani* membrane protein (13, 14, 24; A. C. White, Jr., and D. McMahon-Pratt, Letter, J. Infect. Dis. 161:1313–1314, 1990), provided only partial protection against parasite challenge, and in each instance afforded only a two- to five-fold reduction in the visceral parasite burden.

The limited protection afforded by these strategies prompted us to develop an alternative vaccine approach. This ap-

proach is based on the clinical observation that in areas where the disease is endemic only a small percentage of people who are infected develop active disease. In fact, following infection most individuals have no clinical symptoms yet develop a strong parasite-specific T helper 1 (Th1) cell (gamma interferon [IFN- γ]) response (4) that protects against developing subsequent visceral disease (29). The importance of parasite-specific Th1 responses in protection has also been demonstrated in the murine model of VL (14, 20, 30). Because the subclinically infected population is immune, we hypothesized that a vaccination strategy that leads to the induction of an appropriate cellular immune response would contribute to the control of VL. We further postulated that because immunity is induced by the persistent subclinical infection, the sustained expression of *Leishmania* antigens by immunization with *Leishmania* DNA in a mammalian expression plasmid may mimic this natural immune stimulation. DNA vaccination can induce both humoral and cellular (including major histocompatibility complex class I- and class II-restricted CD8 and CD4 T cells) immune responses (11, 32) and protection against a number of different viral, bacterial, and protozoal pathogens (reviewed in reference 11). Immunization with a large group of antigens encoded by a DNA library (1,000 to 3,000 clones) was effective in vaccinating mice against *Mycoplasma* spp. and *Leishmania major* (5, 23).

We describe here our efforts to develop a DNA vaccine for VL. We used a well-characterized model of VL in which BALB/c mice are infected intravenously (i.v.) with *L. donovani* amastigotes (19, 34). In this model the infection is ultimately controlled without the development of overt clinical symptoms.

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However, the exponential increase in visceral parasite burden during the first 1 to 2 months of infection enabled us to use a vaccine-induced reduction in parasite burden as the endpoint for vaccine efficacy. Our vaccination strategy involved initial immunization with fractions from an *L. donovani* cDNA expression library that totaled approximately 30,000 plasmid constructs. By sequential in vivo DNA immunization and parasite challenge followed by further fractionation of the plasmid pools into smaller groups, we identified a small number of DNA vaccine constructs that induced a strong Th1 response and protection against systemic parasite challenge. To our knowledge, this is the first demonstration that immunization with sequential fractions of a cDNA expression library can successfully lead to identification of individual components of a protective DNA vaccine.

MATERIALS AND METHODS

Parasites. The *L. donovani* 1S strain (MHOM/SD/001S-2D) was used for these studies. Promastigotes were cultured axenically in Medium 199 supplemented with 20% fetal bovine serum and used to prepare soluble *L. donovani* antigen (SLDA) as previously described (19). The strain was continuously maintained by repeated passage through Syrian Golden hamsters. Purified amastigotes were obtained as follows. Spleens from infected hamsters were homogenized in sterile buffer containing 20 mM Na₂PO₄, 104 mM NaCl, 10 mM MgCl₂, 10 mM KCl, 5.5 mM glucose, and 0.5 mM EDTA (pH 7.3) on ice, and the splenic debris and intact spleen cells were removed by multiple centrifugations at $70 \times g$. The amastigote suspension was then passed through a 26-gauge needle and sequentially through three polycarbonate filters (Nucleopore) with pore sizes of 8, 5, and 3 μ m. The amastigotes collected from the last filtration step were washed in Hanks balanced salt solution (HBSS) and counted by phase microscopy in a hemocytometer. The amastigote suspension, which was devoid of host cells, was used immediately for the isolation of RNA or for the mouse infections.

Construction of the cDNA library. We constructed an *L. donovani* cDNA library in the pcDNA3.1 eukaryotic expression vector (Invitrogen) which contains a strong cytomegalovirus promoter. Because it is the amastigote stage of the parasite that persists (and confers immunity) in a subclinical infection, we chose to construct our library from amastigote-derived poly(A)⁺ RNA. Total RNA was extracted from the purified amastigotes using acid-guanidinium isothiocyanate-phenol-chloroform (10). mRNA was purified from the total RNA using the FastTrack isolation system (Invitrogen). The purified mRNA was reverse transcribed using oligo(dT) as a primer, and the cDNA was cloned into the *Eco*RI and *Not*I sites in pcDNA3.1. Transformation of *Escherichia coli* DH5 α strain was accomplished by electroporation to achieve maximum efficiency. We confirmed that the cDNA library was not contaminated by hamster cDNA (the amastigotes were purified from infected hamsters) by Southern blotting (data not shown). Restriction digestion of approximately 70 clones revealed inserts ranging in size from 0.5 to 3 kb, with an average size of approximately 1 kb.

Screening of the library by expression library immunization. The overall schema for identification of vaccine candidate antigens through expression library immunization and sequential fractionation is shown in Fig. 1.

(i) Primary screen. The plasmid cDNA library was cultured on 15 agar plates at a density of approximately 2,000 colonies per plate. Each plate was considered a sublibrary (designated A through O), and a replica was plated onto five nylon filters that were cultured (colony side up) on a Luria-Bertani (LB) agar plate overnight. One of these filters was then transferred to an agar plate supplemented with 20% glycerol, grown for an additional 4 h, and then cryopreserved at -70°C (cryopreserved master plate). Pilot studies showed that by thawing the plate at room temperature for 2 h and then transferring the membrane (colony side up) to a fresh LB-ampicillin plate, the original colonies could be recovered for at least 1 year after cryopreservation. The other nylon filters were replica plated to multiple plates (4 plates per filter, total of 16 plates per sublibrary) and incubated for 14 h, and the bacterial colonies were collected by scraping the agar surface in phosphate-buffered saline (PBS). The bacteria from the replicate plates were then pooled and pelleted, and a portion was frozen as the bacterial pool stock; the remainder was used for plasmid isolation using the Qiagen endotoxin-free plasmid purification system. Endotoxin contamination was measured using a modification of the *Limulus* assay (BioWhittaker), and plasmid DNA used for immunization always had <10 endotoxin units per 100 μ g of DNA. To avoid losing plasmids due to variations in the replication rates that may occur in broth cultures, plasmid DNA was isolated from bacteria cultured on agar plates instead of broth medium.

To determine the protective efficacy of the sublibraries, 4- to 6-week-old BALB/c mice (Charles River Laboratories; four animals per group) were immunized with plasmid DNA (100 μ g in PBS) from each sublibrary by cutaneous (25 μ l per hind foot pad) injection at days 0 and 14. At day 28, the mice were challenged by i.v. (lateral tail vein) injection with 10^6 amastigotes. The splenic and hepatic parasite burden was determined 4 weeks after challenge by quanti-

tative limiting dilution culture (see below). The data are expressed as the mean log reduction in the parasite burden in the liver and spleen in immunized mice compared to mice that were sham vaccinated with DNA from the pcDNA3.1 vector lacking any insert. Statistical analysis was performed using the nonparametric Mann-Whitney U test.

(ii) Secondary screen. The primary screen identified several sublibraries that conferred protection against challenge with *L. donovani* amastigotes. We focused our further studies on two of the protective sublibraries (sublibraries G and O) and used one nonprotective sublibrary (sublibrary B) as a control. With the goal of rapidly narrowing the number of vaccine candidates that needed to be screened, we reasoned that the cDNA clones in the protective sublibraries (G and O) that had complete coding regions were more likely to express a complete, immunogenic protein. To screen the sublibraries G and O for full-length clones, the colonies from the sublibrary were picked with sterile toothpicks from the cryopreserved and thawed master plate and transferred to plates with numbered grids (200 per 150-mm-diameter plate). The colonies from each of the gridded plates were transferred to a nylon membrane (Nytran; Schleicher & Schuell) by overlay impression for 5 min and then placed colony side up on an LB-ampicillin agar plate and incubated overnight. The colonies were lysed and, after drying and UV crosslinking, the membranes were prehybridized and then hybridized with a digoxigenin (Boehringer Mannheim)-labeled probe (4.5 pmol/ml) at 40°C for 4 h. The oligonucleotide probe used (5'-TCAGTTTCTGTACTTTATTG-3') recognizes the *Leishmania* mini-exon sequence which is trans-spliced onto the 5' end of all mature *Leishmania* mRNAs (33). Since the library was constructed using an oligo(dT) primer, the 3' end of the cDNA should have a 3' poly(A) sequence, and thus clones that contained the 5' spliced leader sequence should contain a complete open reading frame (with a variable amount of the flanking 5'- and 3'-untranslated regions [UTRs]). The hybridized membranes were washed twice in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and $0.5\times$ SSC at room temperature, and hybridization was detected by chemiluminescence and autoradiography according to the manufacturer's (Boehringer Mannheim) protocol.

The plasmids containing the full-length cDNAs were isolated from sublibraries G and O. These pools of full-length clones were designated FL-G and FL-O. Plasmid DNA from the FL-G and FL-O pools, the parent sublibraries (G and O), and sublibrary B was isolated, and the immunization experiments were repeated. Groups of eight mice were immunized with the plasmid DNA by either cutaneous (25 μ l per hind foot pad) or i.v. (100 μ l per tail vein) injection at days 0 and 14. Two of the mice were used to determine the vaccine-induced cytokine response before infection, and the remaining six mice were challenged as described above. Control mice were immunized with the pcDNA3.1 vector lacking any insert. The splenic and hepatic parasite burden was determined 4 weeks after challenge as described above.

(iii) Tertiary screen. Because the pool of full-length O plasmids induced protective immunity that was comparable to that with the parent sublibrary, we focused our attention on these clones. The cDNA insert from each of the FL-O plasmids was partially sequenced (in most cases a sequence of 400 to 500 nucleotides was obtained) using a single forward vector-specific primer and an automated, fluorescent DNA sequencer (Model 373A; Applied Biosystems). BLAST was used to search the NCBI databases to identify previously cloned sequences that may have homology to those that we sequenced (2). Where possible, the clones were then grouped according to the type of encoded protein (e.g., all of the plasmids that encoded histone proteins were grouped together) such that five groups, each containing five to nine plasmids, were created (designated FL-O-A, FL-O-B, etc.). Groups of eight mice were immunized by cutaneous injection of 100 μ g of each of the FL-O subpools, and the immunogenicity (cytokine production) and protective capacity of the vaccine subpools was determined as described above.

Determination of parasite burden. Quantitative limiting dilution culture was performed as described previously (25). Each organ was harvested, and its total weight was determined. A weighed piece of spleen or liver (20 to 60 mg) was then homogenized between the frosted ends of two sterile glass slides in 1 ml of complete M199 culture medium and diluted with the same medium to a final concentration of 1 mg/ml. Fourfold serial dilutions of the homogenized tissue suspension were then plated in a 96-well tissue culture plate containing a 50 μ l of blood agar slant and cultured at 26°C for 2 weeks. The wells were examined for motile promastigotes at 3-day intervals, and the reciprocal of the highest dilution which was positive for parasites was considered to be the number of parasites per milligram of tissue. The total organ burden was calculated using the weight of the whole organ.

Vaccine-induced cytokine measurement. Spleens or lymph nodes from control and immunized mice were harvested, and single-cell suspensions were obtained by homogenization of the tissue between the frosted ends of two glass microscope slides. The erythrocytes were lysed with ammonium chloride lysis buffer (Sigma), and the cells were washed and cultured in complete medium (RPMI with 10% heat-inactivated fetal bovine serum, 100 mM glutamine, penicillin-streptomycin, and 5×10^{-5} M 2-mercaptoethanol) at 2×10^6 cells per ml. Cells were cultured in medium alone (control) or stimulated with 5 μ g of concanavalin A (ConA) per ml for 3 days or 25 μ g of SLDA per ml for 2 to 4 days. In some experiments the spleen or lymph node cells were stimulated with washed, viable *L. donovani* promastigotes (10^5 per well). The interleukin-4 (IL-4), IL-10, and IFN- γ levels in the supernatants were determined by sandwich enzyme-linked

FIG. 1. Summary of the expression library immunization approach used to identify *L. donovani* vaccine candidates. For the primary screen, each of 15 sublibraries was studied, but only the protocol for sublibrary A is shown. SL, spliced leader; FL, full-length.

immunosorbent assay (ELISA) using monoclonal antibodies (capture and detection) from Pharmingen (San Diego, Calif.) as described previously (19).

RESULTS

Primary screen of the cDNA library by immunization and challenge. Our first goal was to determine which of the 15 *L. donovani* cDNA sublibraries (totaling approximately 30,000 clones) induced protection against parasite challenge. Mice were immunized with plasmid DNA from these sublibraries and then challenged with parasites. Of the 15 different sublibraries, six induced a statistically significant ($P < 0.03$) reduction in the hepatic parasite burden (Fig. 2). Sublibrary O induced the maximum protection and reduced the hepatic parasite burden by approximately 1,000-fold. There was no statistically significant reduction in the splenic parasite burden in any of the immunized groups compared to the vector control

(data not shown). Also, there was no reduction in visceral parasite burden in mice that received the DNA vector alone compared to unimmunized mice (data not shown).

Secondary screen. The two sublibrary pools (sublibraries G and O) that afforded the most protection and the nonprotective sublibrary B were selected for further study. To isolate clones that had a complete protein coding sequence, bacterial colonies from sublibraries G and O were hybridized with an oligonucleotide that recognized the mini-exon sequence. The sublibraries O and G had approximately 40 full-length clones each (a portion of these plasmids were sequenced from the 5' end and confirmed to contain the spliced leader sequence [data not shown]). Additionally, by an *in vitro* translation system we confirmed that the plasmid constructs could express a protein (data not shown).

The plasmids containing the full-length cDNAs were iso-

FIG. 2. Identification of DNA sublibrary vaccines that protect against systemic challenge with *L. donovani*. Plasmid DNA from 15 sublibraries (designated A to O), each containing approximately 2,000 clones, were screened in vivo by DNA immunization. Groups of four BALB/c mice were immunized with 100 μ g of plasmid DNA from each sublibrary at days 0 and 14 and then challenged i.v. at day 28 with 10^6 amastigotes. The hepatic parasite burden was determined 4 weeks after challenge and is expressed as the mean $\pm$ the standard error of the mean (SEM) log reduction in the parasite burden in the liver of mice that received the sublibrary DNA vaccine compared to mice that received an equivalent amount of only the DNA vector. Vaccine groups whose parasite burden was significantly reduced ($P < 0.03$) are identified by a star.

lated and pooled (designated FL-G and FL-O), and their vaccination efficacy was compared to that of the parent sublibrary. Mice were immunized with sublibraries O and G, sublibrary B (the nonprotective sublibrary), and the full-length pools from sublibraries O and G. For these experiments, we examined the efficacy of these DNAs following local (cutaneous) or systemic (i.v.) immunization. To determine the ability of the vaccine

constructs to induce a cellular immune response, spleen cells were isolated from immunized mice, and cytokine responses were determined following in vitro stimulation with a nonspecific mitogen (ConA) or SLDA. The pattern of cytokine response was similar for both the i.v. and cutaneous routes of immunization. Both the cutaneous and i.v. immunization with the sublibraries O and G and their full-length pools induced strong *Leishmania*-specific IFN- γ production (Fig. 3). Antigen-induced IFN- γ production was substantially higher in the mice immunized with the whole sublibrary O compared to the full-length O fraction. This suggested that immunostimulatory clones were present in the sublibraries that were not present in the full-length pools. The spontaneous release of IFN- γ by spleen cells was not higher in the vaccinated mice compared to unvaccinated mice, indicating that at 2 weeks after the last immunization there was no nonspecific effect of the plasmid DNA. There was no detectable antigen-specific IL-4 or IL-10 response (data not shown). Mice immunized with sublibrary O and the two full-length pools demonstrated approximately a twofold increase in IFN- γ response to the nonspecific mitogen ConA, whereas mice immunized with the nonprotective sublibrary B and the vector control did not (data not shown).

Immunization with the sublibrary fractions G and O and their full-length pools, either by the cutaneous or the i.v. route, induced protection against parasite challenge (Fig. 4). Immunization with the full-length O (FL-O) subpool resulted in the greatest reduction in parasite burden (a 13- to 50-fold reduction in the liver [$P < 0.002$] and a 25- to 65-fold reduction in the spleen [$P < 0.02$]). The sublibrary fraction B, which was not protective in the primary screening immunization, was again nonprotective in this secondary screen, serving as an important negative control. These data, along with the finding of vaccine-induced antigen-specific IFN- γ production, indicated that the protection induced by the sublibraries G and O and their full-length subpools is related to the in vivo expression of *Leishmania* antigens within those pools and not merely to the presence of *Leishmania* DNA in the vector.

Tertiary screen. The protective FL-O pool of plasmids was selected for further study. The cloned inserts from FL-O were partially sequenced and analyzed for similarity to sequences in the GenBank database. A high degree of identity with previ-

FIG. 3. Spleen cell IFN- γ response to SLDA in immunized and control mice. Two BALB/c mice per group were immunized twice by either cutaneous (left panel) or i.v. (right panel) injection of 100 μ g of plasmid DNA (sublibraries B, G, and O; full-length G [FL-G]; full-length O [FL-O]; and vector [V] control) or PBS alone (unimmunized [Un]) at days 0 and 14. At day 28 the mice were sacrificed and the spleen cells isolated and stimulated in vitro with SLDA (25 μ g/ml). Culture supernatants were harvested after stimulation for 96 h, and the concentration of IFN- γ was determined by sandwich ELISA. The concentration of IFN- γ (ng/ml) in the culture supernatants of the unstimulated and SLDA stimulated spleen cells is shown (mean $\pm$ the SEM).

FIG. 4. Protection against *L. donovani* by immunization with DNA sublibrary fractions. Groups of six BALB/c mice were immunized twice by either cutaneous (left panel) or i.v. (right panel) injection of 100 μ g of plasmid DNA (sublibraries B, G, and O; full-length G [FL-G]; full-length O [FL-O]; and vector control) at days 0 and 14. Mice were then challenged i.v. on day 28 with 10^6 amastigotes. The hepatic and splenic parasite burden was determined 4 weeks after challenge and is expressed as the mean ($\pm$ the SEM) log reduction in the parasite burden in the livers and spleens of immunized mice compared to mice that received only the DNA vector. Groups of immunized mice whose parasite burden was significantly reduced ($P < 0.02$) are identified by an asterisk.

ously reported sequences was found in about half of the cases (Table 1).

The plasmids from fraction FL-O were divided into five groups, each containing five to nine unique clones (Table 1). The plasmids were grouped according to similarities of the encoded proteins. Mice were immunized with these plasmid subpools, and the antigen-specific IFN- γ responses were determined. Spleen and lymph node cells from mice immunized with two of the subpools (FL-O-B and FL-O-D) demonstrated strong antigen-induced IFN- γ responses to SLDA and *L. donovani* promastigotes (Fig. 5). Interestingly, for reasons that are not yet clear, the spleen cells responded preferentially to the SLDA, but the LN cells responded better to the whole parasites.

Cutaneous immunization with the subfractions FL-O-B (DNA encoding histone proteins) and FL-O-D (DNA encoding unidentified proteins), the same subfractions that induced an antigen-specific IFN- γ response resulted in an approximately 20-fold reduction in parasite burden in both the liver and the spleen ($P < 0.01$, Fig. 6). Immunization with subfraction FL-O-C also induced a fivefold reduction in splenic parasite burden ($P < 0.01$).

DISCUSSION

We used in vivo immunization, followed by parasite challenge and sequential fractionation steps, to identify a group of recombinant vaccine antigens that induced protection against systemic challenge with *L. donovani*. Starting with a total of approximately 30,000 clones, our vaccination strategy permitted us to define a protective multicomponent DNA vaccine that contained as few as five unique cDNAs. At each step in the fractionation process, the vaccine candidates that induced protection also induced a strong antigen-specific IFN- γ response. To our knowledge, this is the first time a protective expression library vaccine has been characterized to the level of the individual antigens.

The level of protection afforded by our immunization strategy in this model of murine VL was greater than what has been demonstrated using crude parasite extracts or purified proteins (13, 14, 24; White and McMahon-Pratt, Letter). That the vaccine-induced protective effect was more pronounced in the liver compared to the spleen was not surprising since the ac-

quired immunity that develops during the course of infection in this experimental model is more efficient at clearing parasites from the liver than the spleen (19, 34). The approach of immunization with sequential fractions of a DNA expression library was very labor intensive, but our results demonstrate that it is a powerful method for identifying recombinant vaccine candidates. This may be especially helpful for infectious diseases in which protective immunity is mediated by antigen-specific T-cell responses and in which immune sera are unreliable for the identification of relevant antigens in a DNA expression library.

Effective vaccination against *Leishmania* will require the priming of T cells to produce IFN- γ in response to the infecting parasite. Although it is well known that plasmid DNA derived from bacteria acts as a nonspecific adjuvant in the stimulation of a Th1 response (31), several lines of evidence indicate that the protection induced by our multicomponent DNA vaccine was not due to a nonspecific immunostimulatory effect of the vector DNA but rather due to immune responses induced by sequences specific to *Leishmania*. First, the levels of spontaneous release of IFN- γ by spleen or LN cells from vaccinated and unvaccinated mice were similar. Second, the sham-vaccinated (DNA vector control) mice showed no antigen-specific cellular immune response or reduction in parasite burden compared to the unvaccinated controls (data not shown). Third, one of the plasmid sublibraries was consistently nonprotective, indicating that the protection mediated by the multicomponent vaccine was dependent on specific parasite DNA sequences and not just on the presence of plasmid or any *Leishmania* DNA. Fourth, at each step in the DNA library fractionation process, only the protective DNA vaccine pools induced T-cell responses directed specifically at soluble *Leishmania* antigens and whole parasites.

Immunization with the protective DNA library fractions (but not the vector control DNA or the nonprotective DNA library fraction) enhanced the IFN- γ response to the nonspecific mitogen ConA. Most likely, this indicates that the protective sublibraries and their full-length fractions contained antigens that activated T cells so that the response to the second signal was enhanced. Alternatively, antigens expressed by the plasmids may have induced IL-12 production, leading to an increased IFN- γ response to the second stimulus.

TABLE 1. Sequence analysis and fractionation scheme for the full-length O (FL-O) pool

Subpool	Clone	Homology to database sequence
FLO-A	A-1	Ribosomal protein S14
	A-2	Ribosomal protein S13
	A-3	Ribosomal protein S13
	A-4	Ribosomal protein S16
	A-5	Ribosomal protein S18
	A-6	Ribosomal protein L8
	A-7	Ribosomal protein L28
	A-8	Ribosomal protein (fungal)
FLO-B	B-1	Histone 4
	B-2	Histone 4 ^a
	B-3	Histone 3
	B-4	Histone 2B
	B-5	Histone 2B ^b
FLO-C	C-1	<i>L. major</i> D gene
	C-2	<i>L. major</i> D gene ^c
	C-3	No homology
	C-4	No homology
	C-5	No homology
	C-6	No homology
	C-7	<i>L. mexicana</i> short direct repeats
	C-8	Mouse thymosin
	C-9	No homology
FLO-D	D-1	No homology
	D-2	No homology
	D-3	No homology
	D-4	No homology
	D-5	No homology
	D-6	No homology
	D-7	No homology
	D-8	No homology
	D-9	No homology
FLO-E	E-1	<i>Chlamydomonas</i> BBC1 protein
	E-2	<i>T. brucei</i> unidentified sequence
	E-3	No homology
	E-4	No homology
	E-5	No homology
	E-6	No homology
	E-7	No homology
	E-8	No homology
	E-9	No homology

^a Sequence slightly different from FL-O-B-1.^b Sequence slightly different from FL-O-B-4.^c Sequence slightly different from FL-O-C-1.

Contrary to our expectations, sequential fractionation of the protective plasmid pools identified in the primary immunization screen into fractions containing fewer vaccine constructs did not afford greater protection. This suggests that the protection induced by immunization with the larger pools was not mediated by a single antigen since this antigen would have had proportionately greater representation as the sizes of the fractions were reduced. Most likely, the greater protective efficacy of the larger fractions was related to the combined effect of several different antigens. Thus, with sequential fractionation the protective antigens may have been separated and the combined and/or synergistic effect of these antigens diminished. An additional possibility is that the sequential fractionation steps could have separated the antigenic cDNA(s) from plasmids that contained *Leishmania* DNA sequences that had nonspecific immunostimulatory (adjuvant) activity (7). Also, we cannot exclude the possibility that one or more protective cDNAs

were lost in the freezing or thawing of the master plate or in the transfer of the colonies to the gridded plate. Regardless of the reason for the greater protective efficacy of the larger fractions, our findings support the notion that an optimal vaccine is likely to require the inclusion of multiple antigenic epitopes that will direct the host response toward multiple parasite targets. Additionally, a multiantigen vaccine can circumvent the potential limitation of major histocompatibility complex-restricted responses to a particular epitope (12).

We evaluated the immunogenicity and protective efficacy of the multicomponent vaccine when delivered by either the cutaneous or the i.v. route for several reasons. Cutaneous immunization was of interest because the uptake and presentation of vaccine antigens by this route would be targeted to epidermal Langerhans cells or dermal dendritic cells, the most potent antigen-presenting cells. In this regard, we recently demonstrated that delivery of *L. donovani* antigens via dendritic cells induced strong protective immunity against VL (1). Although the i.v. delivery of plasmid DNA has been reported to be variably immunogenic (6, 18), possibly because of plasmid degradation by plasma nucleases (22), we surmised that the delivery of the DNA to the visceral organs might enhance the resistance to visceral *L. donovani* infection. Although we did not evaluate these routes of delivery in a direct comparative experiment, our results indicate that the multicomponent vaccine had similar immunogenicity and protective efficacy when delivered by either the cutaneous or the i.v. route. Further experiments to define the localization and type of the vaccine antigen-presenting cells used during these two modes of delivery are ongoing. Certainly, regardless of the route of delivery, increased targeting of the vaccine antigens to dendritic cells will likely be an important step in optimizing the vaccine efficacy.

We were surprised that immunization with a group of cDNAs that encode histone proteins (subpool FL-O-B) induced a strong Th1 response and protection against parasite challenge. At first glance, histone proteins would not seem to be good vaccine candidates because they are among the most highly conserved proteins and are thought to be targets for the induction of autoimmunity. However, the sequences of our cDNAs (data not shown) and published reports (21, 26, 27) indicate that there is a very low level of homology between the *Leishmania* histone proteins and the mammalian homologues. Several of the *Leishmania* histone proteins have been demonstrated to be targets of the humoral immune response in canine VL (26, 27). This seroreactivity was specific to the *Leishmania* histones, with no cross-reactivity to the mammalian histone homologs. Recognition of histone proteins by T cells has not been reported and, to our knowledge, this is the first evidence that vaccination with histone antigens can effectively protect against an infectious disease.

Another subfraction, FL-O-D, also induced strong *Leishmania*-specific IFN- γ responses and protective immunity. The partial sequences of these novel cDNAs did not reveal significant homology to any DNA sequences in the GenBank database. Further characterization of these cDNAs is in progress.

We chose to immunize with a cDNA rather than genomic library for two reasons. First, a fewer number of clones would have to be screened to achieve reasonable representation of the genome. Second, the spliced leader sequence on the 5' end of mature *Leishmania* mRNAs would facilitate identification of cDNAs containing the complete coding sequence. These full-length cDNAs should encode all the T-cell epitopes and have the initiation codon sequence necessary for expression in eukaryotic cells. Truncated cDNAs may lack an initiation codon or have internal AUGs that would not function as an

FIG. 5. Antigen- and parasite-induced IFN- γ responses of the spleen and lymph node cells in immunized and control mice. Groups of two BALB/c mice were immunized by cutaneous injection of 100 μ g of plasmid DNA from subfractions of the full-length O pool (FLO-A, FLO-B, FLO-C, FLO-D, and FLO-E), vector (V) control, or PBS alone (unimmunized [Un]) at days 0 and 14. On day 28 the mice were sacrificed, and the spleen cells isolated and stimulated in vitro with SLDA (25 μ g/ml) or live *L. donovani* promastigotes (10^5 /well). Culture supernatants were harvested after stimulation for 96 h, and the concentration of IFN- γ was determined by sandwich ELISA. The antigen-induced IFN- γ concentration in culture supernatants is shown as the mean ($\pm$ the SEM) of the stimulated (SLDA and promastigotes) and unstimulated (medium) spleen cells (left panel) and lymph node cells (right panel). The lymph node assay was performed with cells pooled from four popliteal lymph nodes from two mice.

efficient initiation site. Indeed, the full-length cDNAs that we isolated conferred a level of protection comparable to that of the larger sublibraries and therefore drastically reduced the number of clones that needed to be screened to identify the components of a protective vaccine.

It should be noted that these vaccine constructs were protective despite the fact that their sequences may not have been optimal for expression in a mammalian system. Sequence analysis of the *Leishmania* cDNAs that make up the protective multicomponent DNA vaccine demonstrated that the nucleotide sequence in the region of the initiation AUG codon frequently did not conform to the optimal consensus sequence for translation initiation in mammalian cells (17; data not shown). Therefore, it is conceivable that translation of the coding sequence of the DNA vaccine could be enhanced by engineering a mammalian consensus sequence for translation initiation into the *Leishmania* cDNA vaccine constructs. Additionally, the 5'-UTR upstream from a protein coding region may contain nucleotide sequences (especially a high GC content) that may decrease translation and transcription (15, 28), and the 3'-UTR may also diminish expression levels by increasing the instability of mRNAs (16). Analysis of the histone cDNA sequences in our multicomponent vaccine revealed that the 5'- and 3'-UTRs were long and that the 5'-UTRs were GC-rich. The possibility that the removal of these UTRs could increase the level of antigen expression and efficacy of the DNA vaccine is being investigated.

In summary, we have identified a small group of *L. donovani* DNA vaccine constructs that induce a strong Th1 response and confer significant protection against experimental VL. Although the protection is partial, it is substantially greater than what has been achieved previously. We anticipate that the efficacy of the vaccine cDNAs can be enhanced by modifying their sequences to optimize in vivo expression and by more effectively targeting delivery of the vaccine constructs to potent antigen-presenting cells, such as Langerhans cells or dendritic cells. Additionally, the inclusion of a vaccine adjuvant, such as

granulocyte-macrophage colony-stimulating factor or IL-12, may enhance the immunogenicity and efficacy of the vaccine. We are currently investigating these possibilities and are defining the contribution of each of the cDNAs that are part of the multicomponent DNA vaccine. Ultimately, the multicomponent vaccine (or parts thereof) may provide a means to control VL, either through immunization of the at-risk human population or by immunization of the domestic canine reservoir to reduce transmission to humans.

FIG. 6. Protection against *L. donovani* by immunization with DNA subpools of the full-length O fraction. Groups of six BALB/c mice were immunized twice by cutaneous injection of 100 μ g of plasmid DNA from subfractions of the full-length O pool (FLO-A, FLO-B, FLO-C, FLO-D, and FLO-E) and vector control at days 0 and 14. Mice were then challenged on day 28 with 10^6 amastigotes. The visceral parasite burden was determined 4 weeks after challenge and is expressed as the mean ($\pm$ the SEM) log reduction in the parasite burden in the livers and spleens of immunized mice compared to mice which received only the DNA vector. Groups of immunized mice whose parasite burden was significantly reduced ($P < 0.01$) are identified by an asterisk.

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