The Journal of Immunology

Protective Immune Responses Induced by Vaccination with an Expression Genomic Library of *Leishmania major*

David Piedrafita, Damo Xu, David Hunter, Robert A. Harrison and Foo Y. Liew

J Immunol 1999; 163:1467-1472; ;
http://www.jimmunol.org/content/163/3/1467

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Protective Immune Responses Induced by Vaccination with an Expression Genomic Library of *Leishmania major*[1]

David Piedrafita,[2]* Damo Xu,[2]* David Hunter,* Robert A. Harrison,[†] and Foo Y. Liew[3]*

To develop an effective vaccine against the intracellular protozoan parasite *Leishmania* spp., we investigated the feasibility of expression library immunization (ELI) in the mouse. Genomic expression libraries of *L. major* were constructed and used to immunize mice. One of the three libraries (L1, with 10^5 clones) induced a significant protective immune response and delayed the onset of lesion development in highly susceptible BALB/c mice after i.m. immunization, compared with control mice immunized with the empty vector (EV). L1 was then divided into five sublibraries of $\sim 2 \times 10^4$ clones each. Mice immunized with one of the sublibraries (SL1A) developed an even stronger protective effect than that induced by L1. SL1A was further divided into 20 sublibraries (SL2) of $\sim 10^3$ clones each. One of the SL2 libraries (SL2G) induced a strong protective effect against *L. major* infection. In direct comparative studies, the protective effect of the sublibraries was in the order of SL2G > SL1A > L1. Lymphoid cells from mice vaccinated with SL2G produced more IFN-γ and NO, compared with cells from control mice injected with EV. Serum from the vaccinated mice also contained more parasite-specific IgG2a Ab, compared with controls. Therefore, these data demonstrate that ELI is feasible against this complex intracellular parasitic infection, by preferentially inducing the development of Th1 responses. Furthermore, by sequential division of the libraries, this approach may be used to enrich and identify protective genes for effective gene vaccination against other parasitic infections. *The Journal of Immunology,* **1999, 163: 1467–1472.**

The *Leishmania* spp. are dimorphic intracellular protozoa that cause diseases of global impact. World-wide prevalence of leishmaniasis is estimated to be in the region of 12 million cases (1). Chemotherapy has only a modest effect, and there is no effective and safe vaccine against any form of clinical leishmaniasis. However, individuals recovered from clinical leishmaniasis develop strong immunity against reinfection, suggesting that vaccination against leishmaniasis is feasible in principle (reviewed in Ref. 2).

A number of candidate vaccine molecules against experimental murine leishmaniasis have been identified. These include: gp63 (3), gp46 (4), p-4, p-8 (5), thiol-specific antioxidant protein (6), promastigote surface Ag-2 (7), LeIF (8, 9), and lipophosphoglycan associated proteins (10). However, all of these molecules demonstrated only partial protection; some only increase recovery from disease in resistant hosts and often require clinically unacceptable adjuvants.

Genetic vaccination has recently provided a promising new approach to vaccination, and protective responses with DNA vaccines against several pathogens have been demonstrated (reviewed in Refs. 11 and 12). We have shown earlier that the highly susceptible BALB/c mice became more resistant to *L. major* infection following i.m. immunization with a eukaryotic expression plasmid containing cDNA encoding gp63 (13). Subsequently, it was shown that immunization with plasmid expressing the leishmania LACK gene (encoding gp24) induced a strong protective response against *L. major* infection (14). These data suggest the feasibility of DNA vaccination against leishmaniasis.

It is likely that effective vaccination against a complex parasitic infection such as leishmaniasis would require a multivalent vaccine containing a number of candidate genes. Identification of the protective genes may be greatly facilitated by the use of genomic expression library immunization (ELI)[4]. Mice immunized i.m. with a partial genomic library of *Mycoplasma pulmonis* developed significant protective responses against a challenge infection (15). Theoretically, once a protective pool of genomic library of a pathogen is identified, it can be subdivided into smaller pools, eventually leading to the identification of a small number of protective genes.

We have tested this hypothesis using the murine cutaneous leishmaniasis model of *L. major* in BALB/c mice. We report here that mice immunized with a genomic expression library of the parasite developed significant protective immunity against a challenge infection. The strength of the protective effect increased with the sequentially selected sublibraries. Protection was associated with the preferential induction of a specific Th1 cell response. Therefore, these results demonstrate, for the first time, the feasibility of genomic ELI against a eukaryotic parasite. Importantly, they show the potential application of genomic ELI in the identification and characterization of protective genes against clinical parasitic infections.

Materials and Methods

Parasites and mice

Promastigotes of *L. major* (LV39) were passaged and maintained in vitro, as previously described (16). All animals used were 6- to 8-wk-old female BALB/c mice obtained from Harlan Olac (Bicester, U.K.).

*Department of Immunology, University of Glasgow, Glasgow, United Kingdom; and [†]Molecular Biology and Immunology Division, Liverpool School of Tropical Medicine, Liverpool, United Kingdom

Received for publication March 2, 1999. Accepted for publication May 19, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a Wellcome Trust Travelling Fellowship (to D.P.), World Health Organization Global Programme for Vaccines and Immunisation, and the Medical Research Council of U.K.

[2] D.P. and D.X. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. F. W. Liew, Department of Immunology, University of Glasgow, Western Infirmary, Glasgow G11 6NT, U.K. E-mail address: f.y.liew@clinmed.gla.ac.uk

[4] Abbreviations used in this paper: ELI, expression library immunization; gDNA, genomic DNA; EV, empty vector; DTH, delayed-type hypersensitivity; L, library; SL, sublibrary.

0022-1767/99/$02.00

Genomic library construction and purification

Genomic DNA (gDNA) of *L. major* was isolated from stationary-phase promastigotes as previously described (17). gDNA was digested with *Mbol*I to a median size of 0.6 kb and cloned into *Bgl*II and *Bam*HI site of the expression vectors, CMV-GH-F1, CMV-GH-F2, and CMV-GH-F3, in three separate reading frames (kind gifts of Dr. S. A. Johston, University of Texas, Dallas, TX). *Escherichia coli* (DH5α; Life Technologies/BRL, Paisley, U.K.) were transformed and plated onto Luria broth (LB) plates containing ampicillin (100 μg/ml; Sigma, Dorset, U.K.). Several separate stocks consisting of 10^5 transformants for each expression vector were suspended in 1.5 ml LB, containing 50% (v/v) glycerol and stored at −70°C. These stocks were designated *L. major* library 1, 2, and 3 (L1-L3) for each of the transformants cloned into the expression vectors CMV-GH-F1, CMV-GH-F2, and CMV-GH-F3, respectively. Stocks were thawed, amplified overnight in 1.5 liters of LB with ampicillin (100 μg/ml; Sigma), and plasmid DNA was purified from pools of transformants for each library using Qiagen (Sussex, U.K.) kit, according to the manufacturer's specifications. Subsequent partitioning of the *L. major* L1 into smaller groups of clones (sublibraries) was performed by thawing a stock vial, plating the transformants overnight on LB plates containing ampicillin, and resuspending the desired number of transformants in 1.5 ml LB, containing 50% (v/v) glycerol, followed by storage at −70°C. Subsequent plasmid DNA isolation was performed as described above. If one of these sublibraries was protective following vaccination of mice, this stock was again plated on LB plates containing ampicillin, and the same procedure as described above followed.

Immunization and challenge infection

Mice were injected i.m. at two sites in the thigh skeletal muscle with 100 μg of plasmid DNA suspended in 100 μl of saline. In some experiments, mice were injected in their hind footpads with 100 μg of plasmid DNA suspended in 50 μl of saline. Mice were boosted one to three times at 2-wk intervals. Then, 2 wk after the final boost, groups of mice were either sacrificed for immunological assays or infected in one hind footpad with 10^5 stationary-phase promastigotes of *L. major*. Lesion development was measured at regular intervals with a constant-pressure dial caliper (Kroeplin, Munich, Germany) and expressed as the footpad thickness increase of the infected hind foot, compared with the uninfected hind foot. At the end of experiments, mice were sacrificed and infected footpads removed. The number of parasites present in infected footpads were quantified using a limiting dilution method previously described (18). Draining lymph nodes and spleens were harvested for in vitro proliferation and cytokine production.

Cell proliferation and cytokine production

Single cell suspensions were obtained by gentle homogenization. Cells were washed twice with RPMI 1640 (Life Technologies/BRL) and resuspended in RPMI at 5×10^6 cells/ml supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies/BRL), and 50 μM 2-ME (Sigma). Cells were stimulated with either 2 μg/ml anti-CD3 Ab (PharMingen, Kent, U.K.) or *L. major* Ags (18) for up to 96 h. Proliferation assays were performed in triplicates in 96-well culture plates (Nunc, Roskilde, Denmark), with addition of 1 μCi of [^{3}H]thymidine (Amersham Life Sciences, Bucks, U.K.) in 25 μl during the final 16 h of culture before harvesting onto a glass-fiber filter (Packard, Wallac, Milton Keynes, U.K.) using a Micromate 196 Harvester (Packard). [^{3}H]thymidine incorporation was measured using a Matrix 96 direct β counter (Packard). In some experiments, duplicate cultures were performed for up to 96 h and supernatants collected and stored at −70°C until cytokines in the culture supernatant were determined by ELISA using paired Ab (PharMingen), as previously described (19). The lower limit of detection of the assays was as follows: murine IFN-γ, 10 pg/ml; murine IL-4, 40 pg/ml.

Measurement of delayed-type hypersensitivity (DTH), NO, and Ab

Groups of mice were injected in the right hind footpad with 50 μl of PBS containing killed *L. major* Ags (equivalent to 10^6 promastigotes). Footpad swelling was measured at regular intervals for up to 72 h with a constant-pressure dial caliper (Kroeplin). Ab against *Leishmania* was measured by an ELISA method using 96-well plates coated with soluble *L. major* Ags, as previously described (20). Rabbit anti-mouse IgG1 and IgG2a Abs were obtained from PharMingen. Nitrite was measured by the Greiss method (21).

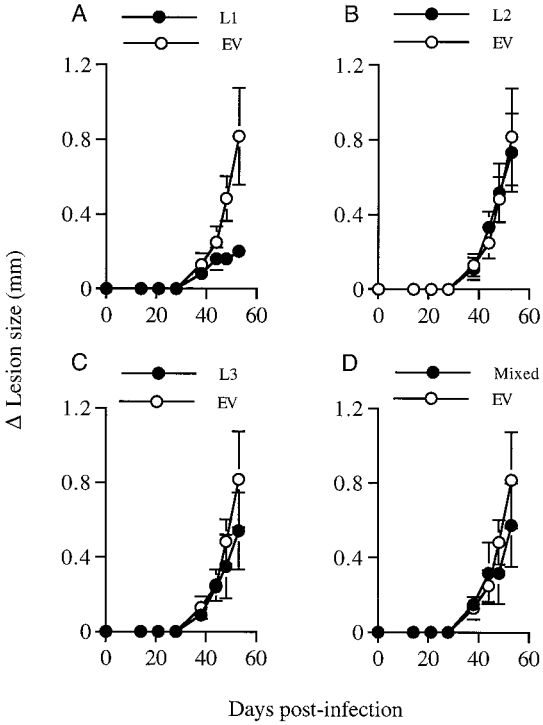

FIGURE 1. Vaccination with genomic expression libraries of *L. major*. Groups of BALB/c mice were immunized and boosted twice i.m., 2 wk apart, with 100 μg of each of the expression libraries L1, L2, L3 ($\sim 10^5$ clones/library), or an equimolar mixture of the three libraries (mixed), or the control empty vector (EV). The animals were challenged 2 wk after the last immunization with 10^5 stationary-phase *L. major* promastigotes in the footpad. Disease progression was measured as footpad swelling. Data are mean change in lesion size ± SEM, $n = 7$. Similar results were obtained in three independent experiments. Mice immunized with EV or injected with PBS developed indistinguishable lesions (data not shown).

Statistical analysis.

Statistical significance ($p < 0.05$) of differences between treatment groups was determined by a one-way ANOVA and the Student's *t* test.

Results

Vaccination with L. major *genomic library*

To capture the majority of the parasite Ags, gDNA of the entire *L. major* promastigote was digested and cloned into three different frames of the expression vector, CMV-GH-F1, CMV-GH-F2, and CMV-GH-F3 (15), and three expression libraries (L1, L2, and L3) were prepared, each containing $\sim 10^5$ clones. Groups of BALB/c mice were immunized i.m. in the thigh with 100 μg plasmid DNA of L1, L2, L3, or a combination of the three (mixed). Control mice were injected with the empty vector (EV) or PBS. The vaccination was repeated three times at 2-wk intervals and challenged in the footpad with 10^5 stationary phase *L. major* promastigotes 2 wk after the last immunization. Lesion development was measured at regular intervals. Mice vaccinated with L1 developed smaller lesions than the controls injected with EV (Fig. 1*A*). Mice immunized with L2, L3, or a mixture of the three libraries developed lesions indistinguishable from mice injected with EV (Fig. 1, *B–D*). No difference in the lesion development was detected between mice vaccinated with EV or PBS alone (data not shown), showing that the plasmid alone containing bacterial DNA did not induce a significant degree of protection. Additional experiments showed that similar results were obtained when mice were given two instead of three immunizations (data not shown).

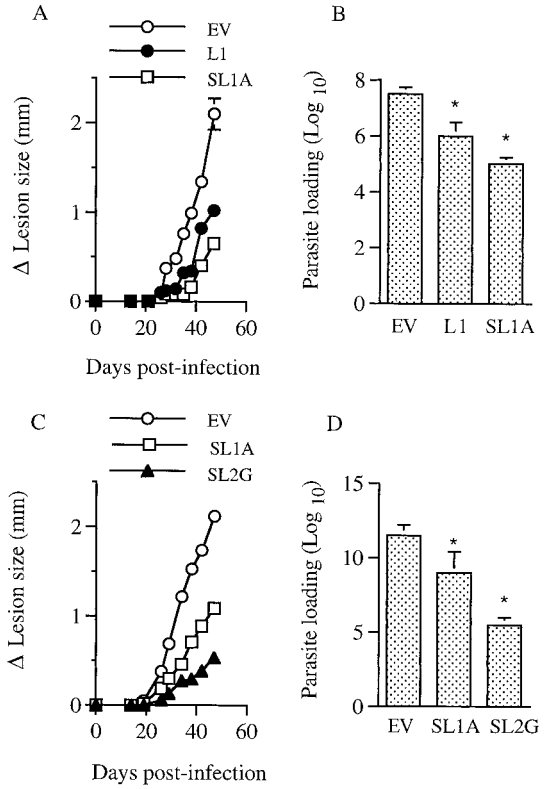

FIGURE 2. Vaccination with sibling libraries of L1. Groups of mice were immunized and boosted with five sibling libraries of L1, SL1A-SL1E ($\sim 2 \times 10^4$ clones/library) or L1 or EV and challenged as in Fig. 1. Mice immunized with SL1A developed smaller lesions (*A*) and lower parasite loads (*B*) in the infected footpad, compared with mice immunized with L1 or EV. SL1A was further subdivided into 20 sibling libraries, SL2A-SL2T ($\sim 10^3$ clones/library). Groups of mice were immunized and boosted i.m. with each of the SL2 libraries and challenged as above. Of the 20 SL2 sibling libraries, only SL2G induced significantly smaller lesions (*C*) and parasite loads (*D*), compared with SL1A. Mice immunized with the rest of the SL2 libraries developed lesions either similar to mice immunized with SL1A (15/20) or EV (4/20) (data not shown). Experiments were terminated on day 50 postinfection, when the lesions in the EV group began to ulcerate, as required by guideline of the Home Office, U.K. Results are expressed as mean ± SEM, $n = 7$, and are representative of three experiments. *, $p < 0.05$, compared with the EV group.

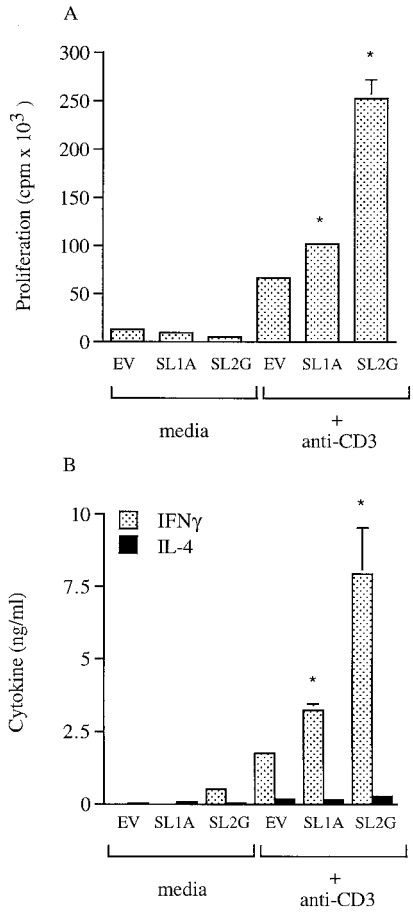

FIGURE 3. Proliferative response and cytokine production by T cells from mice immunized with the genomic libraries of *L. major*. Groups of mice were immunized and boosted twice, 2 wk apart, with SL2G, SL1A, or EV. Draining lymph node cells were collected 2 wk after the last injection and cultured with anti-CD3 Ab. T cell proliferation was determined by thymidine incorporation (*A*) at 96 h. IFN-γ and IL-4 production (*B*) was determined in the culture supernatant (24 h) by ELISA. Data are mean ± SD, $n = 5$, and are representative of two experiments. *, $p < 0.05$, compared with the EV group. Similar results of cytokine production were obtained in supernatants collected at 48 and 72 h.

Vaccination with genomic sublibraries

To enrich the protective genes, L1 was partitioned into five sublibraries (SL1A-SL1E) containing $\sim 2 \times 10^4$ clones each. Groups of mice were then vaccinated with the purified plasmids from each of the sibling libraries, and one of the sibling libraries (SL1A) showed a further decrease in footpad lesion development following a challenge infection, compared with mice vaccinated with L1 (Fig. 2*A*). By day 50 postinfection, the lesions in the group injected with EV began to ulcerate, and the experiments were terminated as required by the guidelines of the animal experimentation of the Home Office, U.K. At this stage, mice vaccinated with L1 or SL1A had a mean footpad parasite load 1.5 and 2.5 $\log_{10}$ lower than that of the control group immunized with EV, respectively (Fig. 2*B*).

We then further divided SL1A into 20 smaller sublibraries (SL2A-SL2T), each containing $\sim 10^3$ clones. Groups of mice were then vaccinated with purified plasmid from each of the SL2 groups and their ability to induce protective immunity examined. Control mice were vaccinated with SL1A or EV. Of the 20 groups of mice vaccinated with the SL2 libraries, 4 had lesions similar to those of control EV-injected mice, 15 had lesions not significantly different from those vaccinated with SL1A, and 1 (SL2G) showed a further decrease in lesion development compared with the SL1A-vaccinated mice (Fig. 2*C*). Mice vaccinated with SL1A or SL2G also contained 3.5 and 6 $\log_{10}$ lower parasite loads compared with mice vaccinated with EV, respectively (Fig. 2*D*). The protective effect is systemic and not localized, since mice immunized and boosted in the right foot and challenged in the right or the contralateral left footpad exhibited similar levels of protection (data not shown). Furthermore, similar results were obtained whether SL2G was delivered i.m. in the thigh or injected s.c. in the footpad (data not shown).

Immunological responses induced by expression library immunization

Anti-CD3 activation. Mice vaccinated with SL1A or SL2G were sacrificed 2 wk after the third immunization, and draining lymph node cells were collected and cultured with immobilized anti-CD3 Ab (polyclonal T cell activation) in vitro. Cells from mice immunized with SL2G produced significantly stronger proliferation and secreted more IFN-γ than cells from mice immunized with SL1A, which, in turn, showed a stronger proliferative response and produced more IFN-γ than cells from the control mice injected with

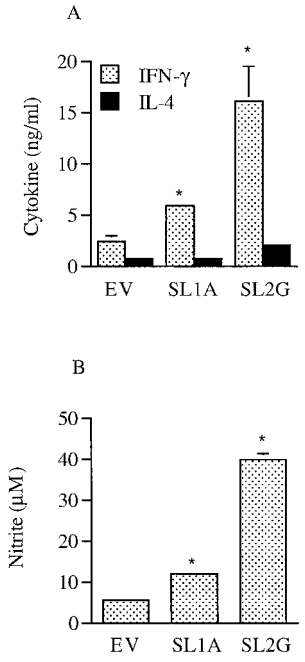

FIGURE 4. IFNγ, IL-4, and NO production by cells from vaccinated mice stimulated with leishmanial Ags. Groups of mice were immunized and boosted with SL2G, SL1A, or EV and challenged with *L. major*, as in Fig. 1. Draining lymph node cells were collected 5 wk after challenge infection and cultured with soluble *L. major* Ags. Supernatant was collected 48 h later and assayed for IFN-γ, IL-4 (*A*), or nitrite (*B*). Data are mean ± SD, $n = 5$, and are representative of two experiments. ∗, $p < 0.05$, compared with the EV group.

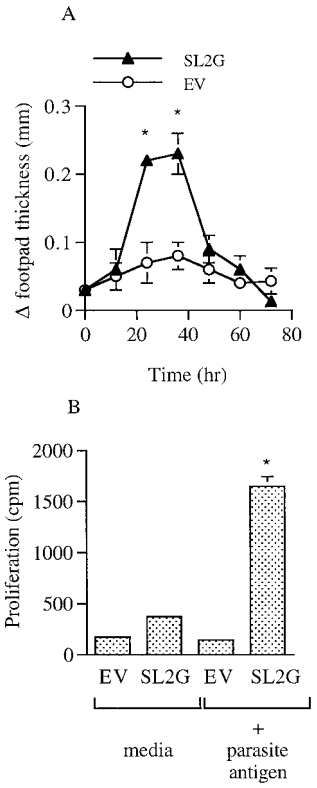

FIGURE 5. DTH and T cell proliferation of mice immunized with SL2G. Groups of mice were immunized and boosted with SL2G or EV. Two weeks later, they were injected in the right hind footpad with killed *L. major* and footpad swelling determined over 72 h (*A*). Data are means ± SEM, $n = 5$, representative of two experiments. ∗, $p < 0.05$, compared with the EV group. Killed parasites were used, as these will avoid any footpad swelling due to infection with live parasites. Draining lymph node cells were collected from other immunized and boosted mice and cultured with soluble *L. major* Ags. T cell proliferation (*B*) was determined by thymidine uptake 96 h later. Data are mean ± SD, $n = 5$, representative of two experiments. ∗, $p < 0.05$, compared with the EV group. The data were not subtracted from background cpm. The stimulating index of the SL2G group was 10.2.

EV (Fig. 3, *A* and *B*). In contrast, cells from all three groups of mice produced low and indistinguishable levels of IL-4. No proliferative response or cytokine production was detected when cells were cultured with leishmanial Ags, presumably the level of specific T cell response induced by the libraries before a challenge infection was too low to be detected by the in vitro assays.

***Leishmania*-specific response.** Groups of mice were immunized and boosted i.m. with 100 μg of SL1A, SL2G, or EV and challenged in the footpad with 10^5 *L. major* promastigotes. Five weeks later, the mice were sacrificed, and draining lymph node cells were cultured with soluble *L. major* Ags in vitro. Supernatants were collected at 48 h and assayed for IFN-γ, IL-4, and nitrite concentrations. Cells from mice immunized with EV produced minimum amounts of the cytokines or NO. In contrast, cells from mice immunized with SL1A produced significant amounts of IFN-γ and NO, which were markedly enhanced in the cultures of cells from mice immunized with SL2G (Fig. 4, *A* and *B*). All cultures produced modest amounts of IL-4. Similar results were obtained with spleen cells (data not shown). Thus, mice immunized with plasmid DNA of SL2G developed significant Th1 responses and produced substantial amounts of NO.

We then investigated the ability of SL2G to induce DTH and T cell proliferation against *L. major* Ags before a challenge infection. Mice were immunized and boosted i.m. with 100 μg of SL2G or EV. Two weeks later, some mice were injected in the footpad with 10^6 killed *L. major* promastigotes and the footpad swelling measured over the following 72 h. Other mice were sacrificed and draining lymph node cells collected for culturing with soluble *L. major* Ags in vitro. Mice immunized with SL2G developed substantial DTH to leishmanial Ags, compared with mice injected with EV (Fig. 5*A*). Similarly, cells from mice immunized with SL2G produced significantly stronger specific T cell proliferation, compared with cells from mice injected with EV (Fig. 5*B*).

Serum Ab. Mice were immunized and boosted with 100 μg of SL2G or EV and challenged 2 wk later with *L. major* promastigotes. Sera were collected just before or 5 wk after the challenge infection and assayed for leishmanial-specific IgG1 and IgG2a Abs. Sera from mice immunized with SL2G contained markedly elevated specific IgG2a Ab before and after the challenge infection, compared with sera from mice injected with EV (Fig. 6). In contrast, specific IgG1 Ab was not detectable before infection and was indistinguishable between the two groups of mice following infection. This is consistent with the results obtained above that SL2G preferentially induced a Th1 response.

Pathology

To determine whether genomic ELI induced long-term pathology, groups of mice were immunized and boosted i.m. with 100 μg of L1, EV, or PBS and were observed over a 2-yr period. All of the mice lived normally and showed no sign of ill health. Histological examinations of tissue sections at the sites of vaccination over this period showed no sign of pathology, compared with control mice injected with PBS (data not shown).

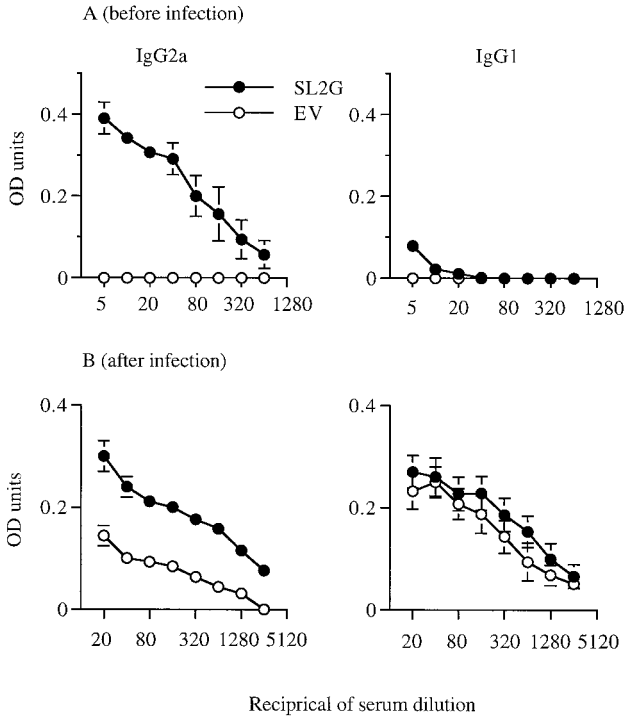

FIGURE 6. Specific Ab production by mice vaccinated with SL2G. Groups of mice were immunized and boosted with SL2G or EV and challenged 2 wk later with *L. major*. Sera samples were collected immediately before (*A*) or 5 wk after (*B*) infection and assayed for leishmanial-specific IgG2a or IgG1 Abs. Data are presented as OD units against reciprocal serum dilution and are mean ± SD of five individual mice.

Discussion

Since the early demonstration of genomic ELI as a feasible approach against the prokaryote *M. pulmonis* (15), there has been no report of successful vaccination using the same approach against a more complex eukaryotic pathogen. It is generally held that genomic ELI may not be applicable to more complex parasites. This is because the large genomes of these parasites may preclude the success of such immunization, due to the extremely small amounts of protective proteins likely to be presented to the immune system, represented in the starting pool of the expression library (22). Data presented here demonstrate that genomic expression library immunization is indeed feasible against the complex intracellular parasite, *L. major*. Furthermore, by successively subdividing the library and selecting the protective library in vivo, the process was capable of enriching the protective genes/gene fragments, leading to incremental improvement in the protective effect induced. The protection was associated with the preferential induction of a Th1 response with elevated IFN-γ and IgG2a production and NO synthesis.

We have demonstrated earlier that vaccination with plasmid encoding gp63 also preferentially induced a Th1 response (13). Others have shown that immunization with leishmanial DNA encoding LACK induced a protective Th1 response (14), whereas injection with the LACK protein promoted the disease-exacerbating Th2 response against *L. major* infection (23). Thus, DNA vaccination holds considerable promise for vaccination against diseases in which Th1 responses are desirable. However, the route of vaccination appears to be of critical importance. In early experiments, we compared s.c. and i.m. immunization with that of subdermal immunization using the gene gun method (24). We found that mice immunized and boosted with 100 μg of L1 injected s.c. or i.m. consistently showed significantly improved resistance against *L. major* infection, whereas, mice immunized and boosted with 20 ng of L1 delivered by the gene gun did not (data not shown). This is consistent with an earlier report that DNA delivered by the gene gun preferentially induced Th2 responses (25). It is noteworthy that i.m. immunization induced systemic immunity because vaccination was equally protective whether the challenge infection was administered in the same footpad as the DNA injection or at the contralateral foot. While there is no significant difference whether two or three immunizations were administered, immunization with one injection alone was insufficient to induce a detectable degree of protection (data not shown). The increased protective effect and Th1 responses when vaccinated with the successive smaller libraries could represent the enrichment of the protective genes/gene fragments. It is of interest to note that while some of the sibling libraries were not protective, none of them was diseases exacerbative. Within the limitation of the assay used, this argues against the presence of a dominant disease-promoting gene in the system used here.

The fact that there was no significant difference in disease development in mice injected with EV or PBS also argues against a protective effect of the bacterial CpG motif in the expression plasmid in our system. However, it should be noted that the effect of CpG tends to be short-lived and requires the simultaneous administration of CpG with the immunizing Ags (26). In our system, mice were challenged 14 days after the last boosting, by which time any effect of CpG would have waned. In addition, of the three expression libraries (L1-L3), which were cloned into three different frames of the expression vector, only L1 showed any detectable protective effect. This result also supports the leishmanial-specific nature of the immunization and argues against the notion that the protective effect was mediated by nonspecific sequences of leishmanial oligonucleotides. The use of a genomic library, rather than a cDNA, library has the advantage of avoiding the selection of stage-specific genes or genes with a high level of expression. Thus, the protective genes enriched, and ultimately identified, could be effective against the promastigote and the amastigote stages of the parasite. Furthermore, the use of the same strong promoter (CMV) would ensure the equal expression of the cloned genes, whatever the levels of their expression in the parasite.

The genome size of *Leishmania* spp. has been estimated to be $\sim 5 \times 10^7$ bp with 25% of the gDNA made up of various noncoding forms of repetitive sequences (27). However, due to the absence of introns in the parasite genome, the construction of the initial library L1 is likely to represent most of the coding sequences of *L. major*. In addition, it is likely that the protective clones identified in this study represent protective epitopes rather than full-length proteins, as the ~600-bp sequences cloned into the expression vectors would more often represent gene fragments rather than full-length genes of *L. major*. While sterile immunity has not been achieved so far, the incremental protective effect with successive subdivision of the libraries into smaller pools of clones is encouraging. The degree of protection achieved so far, determined by lesion development and parasite load, is at least comparable to those attained with single protein Ags (3–9), the whole soluble leishmanial Ags (data not shown), or gp63 DNA. Thus, the method described here has the potential of enriching and identifying novel protective genes for effective vaccination against leishmaniasis. A full characterization of the protective immune responses will be reported with the identification of the protective genes.

Finally, the vaccination schedule appears not to induce any sign of clinical abnormality or local pathology over a 24-mo period

studied. This, together with the potential of identifying and characterizing a small number of protective genes, places the process of sequential selection of protective libraries described in this report in a promising position in developing an effective DNA vaccine against leishmaniasis. The general applicability of this approach to other complex parasitic infections remains to be explored.

References

1. Modabber, F. 1987. The leishmaniases. In *Tropical Disease Research, a Global Partnership, Eighth Programme Report, TDR*. J. Maurice and A. M. Pearce, eds. World Health Organization, Geneva. pp. 99–112.
2. Liew, F. Y., and C. A. O'Donnell. 1993. Immunology of leishmaniasis. *Adv. Parasitol. 32:161.*
3. Russell, D. G., and J. Alexander. 1988. Effective immunization against cutaneous leishmaniasis with defined membrane-antigens reconstituted into liposomes. *J. Immunol. 140:1274.*
4. Champsi, J., and D. McMahon-Pratt. 1988. Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. *Infect. Immun. 56:3272.*
5. Soong, L., S. M. Duboise, P. Kima, and D. McMahon-Pratt. 1995. *Leishmania pifanoi* amastigote antigens protect mice against cutaneous leishmaniasis. *Infect. Immun. 63:3559.*
6. Webb, J. R., A. Campos-neto, P. J. Ovendale, T. I. Martin, E. J. Stromberg, R. Badaro, and S. G. Reed. 1998. Human and murine immune responses to a novel *Leishmania major* recombinant protein encoded by members of a multicopy gene family. *Infect. Immun. 66:3279.*
7. Handman, E., F. M. Symons, T. M. Baldwin, J. M Curtis, and J. Y. Scheerlinck. 1995. Protective vaccination with promastigote surface antigen 2 from *Leishmania major* is mediated by a Th1 type of immune response. *Infect. Immun. 63: 4261.*
8. Skeiky, Y. A., J. A. Guderian, D. R. Benson, O. Bacelar, E. M. Carvalho, M. Kubin, R. Badaro, G. Trinchieri, and S. G. Reed. 1995. A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin-12. *J. Exp. Med. 181:1527.*
9. Skeiky, Y. A., M. Kennedy, D. Kaufman, M. M. Borges, J. A. Guderian, J. K. Scholler, P. J. Ovendale, K. S. Picha, P. J. Morrissey, K. H. Grabstein, A. Campos-Neto, and S. G. Reed. 1998. LeIF: a recombinant *Leishmania* protein that induces an IL-12-mediated Th1 cytokine profile. *J. Immunol. 161:6171.*
10. Russo, D. M., S. J. Turco, J. M. Burns, and S. G. Reed. 1992. Stimulation of human T lymphocytes by *Leishmania* lipophosphoglycan-associated proteins. *J. Immunol. 148:202.*
11. Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. *Annu. Rev. Immunol. 15:617.*
12. Tighe, H., M. Corr, M. Roman, and E. Raz. 1998. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol. Today 19:89.*
13. Xu, D., and F. Y. Liew. 1995. Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of *L. major*. *Immunology 84:173.*
14. Gurunathan, S., D. L. Sacks, D. R. Brown, S. L. Reiner, H. Charest, N. Glaichenhaus, and R. A. Seder. 1997. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers significant protective immunity to mice infected with *Leishmania major. J. Exp. Med. 186:1137.*
15. Barry, M. A., W. C. Lai, and S. A. Johnston. 1995. Protection against mycoplasma infection using expression-libray immunisation. *Nature 377:632.*
16. Liew, F. Y., J. G. Howard, and C. Hale. 1984. Prophylactic immunisation against experimental leishmaniasis. III. Protection against fatal *Leishmania tropica* infection induced by irradiated promastigotes involves Lyt-$1^{+}2^{-}$ T cells that do not mediate cutaneous DTH. *J. Immunol. 132:456.*
17. Kelly, J. M. 1993. Isolation of DNA and RNA from *Leishmania. Methods Mol. Biol. 21:123.*
18. McSorley, S. J., D. Xu, and F. Y. Liew. 1997. Vaccine efficacy of *Salmonella* strains expressing glycoprotein 63 with different promoters. *Infect. Immun. 65: 171.*
19. McInnes, I. B., B. Leung, X. Q. Wei, C. C. Gemmell, and F. Y. Liew. 1998. Septic arthritis following *Staphylococcus aureus* infection in mice lacking inducible nitric oxide synthase. *J. Immunol. 160:308.*
20. Yang, D. M., N. Fairweather, L. Button, W. R. McMaster, L. P. Kahl, F. Y. Liew. 1990. Oral *Salmonella typhimurium* (AroA) vaccine expressing a major leishmanial surface protein (gp63) preferentially induced Th1 cells and protective immunity against leishmaniasis. *J. Immunol. 145:2281.*
21. Kolb, J. P., N. Paul-Eugene, C. Damais, K. Yamaoka, J. C. Drapier, and B. Dugas. 1994. Interleukin-4 stimulates cGMP production by IFN-γ-activated human monocytes: involvement of the nitric oxide synthase pathway. *J. Biol. Chem. 269:9811.*
22. Ulmer, J. B., and M. A. Liu. 1996. ELI's coming: expression library immunization and vaccine antigen discovery. *Trends Microbiol. 4:169.*
23. Julia, V., M. Rassoulzadegan, and N. Glaichenhaus. 1996. Resistance to *Leishmania major* induced by tolerance to a single antigen. *Science 274:421.*
24. Tang, D., M. DeVit, and S. A Johnson. 1994. Genetic immunisation is a simple method for eliciting an immune response. *Nature 356:152.*
25. Fuller, D. H., and J. R. Hayes. 1994. A qualitative progression in HIV type-1 glycoprotein-120-specific cytoxic cellular and humoral immune-responses in mice receiving a DNA-based glycoprotein-120 vaccine. *AIDS Res. Hum. Retroviruses 10:1433.*
26. Zimmermann, S., O. Egeter, S. Hausmann, G. B. Lipford, M. Rocken, H. Wagner, and K. Heeg. 1998. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine Leishmaniasis. *J. Immunol. 160: 3627.*
27. Kelly, J. M. 1993. Isolation of DNA and RNA from *Leishmania. Methods Mol. Biol. 21:123.*