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Vaccine 21 (2003) 2161–2168

Vaccine

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DNA vaccines induce partial protection against *Leishmania mexicana*

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Received 11 February 2002; received in revised form 31 October 2002; accepted 5 November 2002

Abstract

As part of an ongoing effort to develop a vaccine against *Leishmania mexicana*, we tested DNA vaccines encoding *L. mexicana* GP63, CPb, and LACK, and *L. amazonensis* GP46, to evaluate this strategy and define the best antigen candidates. Immune responses and vaccine efficacy were evaluated in BALB/c mice immunized with plasmid DNA encoding the different antigens. All four DNA vaccines induced *Leishmania*-specific humoral and lympho-proliferative immune responses. However, only mice immunized with VR1012-GP46, VR1012-GP63 and VR1012-CPb were partially protected against infection, as evidenced by reduced lesion size and parasite burden. Interestingly, immunization of mice with a mixture of these three plasmids further increased protection. Thus, plasmids encoding CPb, GP63 and GP46 represent good candidates for further development of DNA vaccines against *L. mexicana*.

Keywords: *Leishmania mexicana*; DNA vaccines; Partial protection

1. Introduction

Leishmaniasis is caused by some of the 30 species of protozoan parasites from the *Leishmania* genus, from which about 20 are pathogenic for humans. They are obligate intracellular parasites of host macrophages and cause diverse clinical manifestations ranging in severity from self-healing cutaneous ulcers to chronic diffuse mucocutaneous lesions or even a fatal disseminated visceral form. *Leishmania mexicana* is the major cause of leishmaniasis in southern Mexico and parts of central America. This species causes localized cutaneous leishmaniasis, locally known as chiclero's ulcer, but may also induce more severe forms of the disease such as the diffuse, mucosal or visceral forms.

Because of the difficulties associated with therapeutic treatment, and the common occurrence of irreversible scars upon healing of the lesions, extensive efforts have been devoted to the development of a vaccine [1]. Immunization with killed or live-attenuated parasites demonstrated the feasibility of a vaccine [2,3], but the limitations of such vaccines indicated the need for more defined preparations. Most vaccine studies have focused on *Leishmania major* as a model for vaccine development, and have shown that protection relied strongly on the induction of a T-helper 1 (Th1) cell type response [4]. Indeed, cytokines produced by Th1 cells can activate macrophages and lead to parasite killing, whereas cytokines produced by Th2 cells have the opposite effect and thus exacerbate the development of lesions [4]. Both $CD4^+$ and $CD8^+$ T-cells have been shown to be critical for protection.

A large number of *L. major* antigens have been identified, and tested as recombinant protein vaccines. Most of them induced limited levels of protection against subsequent infection [5–10]. DNA vaccines encoding some of these antigens have also been tested. This approach is based on the direct introduction of a plasmid DNA encoding an antigenic protein into host cells in vivo. The endogenous expression of a foreign antigen may induce strong antibody production as well as a complete cell-mediated immune response [11]. Because DNA vaccines have a strong Th1 bias in the immune response they induce, they appear particularly promising in the case of *Leishmania*. Indeed, the superior efficacy of DNA vaccines encoding GP63, PSA-2 and LACK, compared to their recombinant counterparts, demonstrated the potential of this approach [12–16]. As expected, this immune protection was associated with the induction of a Th1 type response in immunized mice.

In contrast, the control of *L. mexicana* infection is potentially more difficult than that of *L. major*, as this species

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0264-410X/02/$ – see front matter © 2002 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0264-410X(02)00769-7

seems particularly adept at subverting a protective immune response into a non-protective one [17–19]. Indeed, most mouse strains are extremely susceptible to *L. mexicana* infection and develop non-healing lesions, whereas they are comparatively resistant to *L. major* infection.

Purified or recombinant antigens such as GP63, cysteine proteinase (CP) b and GP46/M-2 can induce significant protection against *L. mexicana* or its close relative *L. amazonensis* [10,20–22]. However, as in the case of *L. major*, the efficacy of these vaccine candidates is still partial, and DNA vaccines may be able to induce a better immunity than recombinant vaccines. In this study, we thus tested the immunogenicity and efficacy of DNA vaccines encoding *L. mexicana* antigens GP63, GP46, CPb, and LACK, to determine the usefulness of a DNA vaccine approach, and define the most promising antigens against *L. mexicana*.

2. Material and methods

2.1. Plasmid construction

The cDNAs encoding *L. mexicana* GP63 and CPb, and *L. amazonensis* GP46/M-2 were generous gifts from Dr. D. Russell (Washington University, St. Louis, MO, USA), Dr. J. Mottram (University of Glasgow, UK), and Dr. D. McMahon-Pratt (Yale University, New Haven, CT, USA), respectively. For p36/LACK, we used the *L. major* cDNA, generously provided by Dr. N. Glaichenhaus (University of Nice, Valbonne, France), which was latter reported to be identical to the *L. mexicana* corresponding sequence [23]. The VR1012 vector was generously provided by Vical Inc. (San Diego, CA, USA). Expression of cDNAs in this vector is under the control of a cytomegalovirus (CMV) promoter. Complementary DNAs were subcloned into the VR1012 vector using standard molecular biology techniques as described previously [24] and sequenced. Endotoxin-free plasmids were purified from *E. coli* cultures using Qiagen EndoFree plasmid purification kits (<100 EU/mg), and quantified by spectrophotometric analysis at 260 nm. In addition, the quality of the plasmids was evaluated by restriction enzyme digestion and agarose gel analysis, as well as spectrophotometric analysis at 230, 280 and 325 nm. The ratio OD_{260}/OD_{280} was in the range of 1.8–2.0.

2.2. DNA immunization

Four–six-week-old female BALB/c mice were used in all experiments. Mice were immunized with 100 μg of plasmid DNA in 100 μl saline solution in the quadriceps, and boosted 2 weeks later by a second injection. Control mice received an identical amount of the VR1012 vector or saline solution. In latter experiments, mice were immunized with a mix of three plasmids (VR1012-GP63, VR1012-CPb and VR1012-GP46) at a dose of 50 μg of each plasmid in a total of 100 μl, and boosted 2 weeks latter as previously.

2.3. Antibody response

Two–three weeks after the last plasmid DNA injection, we measured total IgG titers in serial dilutions of serum samples by enzyme-linked immunosorbent assay (ELISA) [24]. Soluble *Leishmania* antigen (SLA) was prepared from two distinct *L. mexicana* strains: a reference strain (MNYC/BZ/62/M379), and a strain recently isolated in the state of Campeche, Mexico (MHET/MEX/97/Hd18, provided by Dr. Fernando Andrade, Universidad Autónoma de Yucatán, Mérida, Mexico). Briefly, late log phase *L. mexicana* parasites were collected by centrifugation, washed with phosphate buffer saline (PBS) four times, and resuspended in a 100 mM Tris, pH 7.3 buffer with 1 mM EDTA, 0.5 mM PMSF and 2.5 μg/ml Leupeptin. Parasites were sonicated, and centrifuged for 20 min at 15,000 × *g*. The supernatant was centrifuged again for 4 h at 39,000 × *g*, and the supernatant was dialized against 2–4 l of cold PBS overnight with several changes of buffer. The SLA was then filtered for sterilization, and 96 wells microplates (Costar, USA) were coated overnight with 0.5 μg of SLA per well. After incubation with various serum dilutions and alkaline phosphatase-labeled anti-mouse secondary antibody (GIBCO BRL, USA), the phosphatase activity was detected using *p*-nitrophenyl phosphate (Sigma, USA) as substrate. Plates were read at 405 nm (Bio-Rad 550 reader), and titers were determined as the reciprocal of the dilution required to reach half-maximum binding. For IgG subtype analysis, a 1:100 serum dilution was tested similarly using anti-mouse IgG1 and IgG2a secondary antibodies (Serotec, UK), respectively.

2.4. Lymphoproliferative response

Two–three weeks after the last immunization, some mice were sacrificed, spleen cells were collected, and 4×10^5 cells per well were plated in DMEM medium supplemented with 10% fetal serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 50 μM ß-mercaptoethanol (all from GIBCO, USA). Spleen cells from individual mice were then stimulated with 0.5, 5.0, and 25.0 μg/ml of M379 or Hd18 strains SLA for 4 days, in triplicate wells. [^{3}H]thymidine (0.5 μCi per well) was added for the last 24 h, and then cells were harvested. Incorporated [^{3}H]thymidine was evaluated by liquid scintillation counting. Stimulation index were calculated as the ratio of stimulated/unstimulated incorporation of [^{3}H]thymidine. Control cells were stimulated with 2 μg/ml of Concanavalin A.

2.5. DNA vaccine efficacy

Two–three weeks after the last DNA immunization, mice were challenged by the injection in the footpads of 2×10^6 stationary phase *L. mexicana* promastigotes of the Hd18 strain. The time course of infection was monitored by measuring the length and width of footpad swelling with a

Vernier caliper for up to 12 weeks. Mice were sacrificed at 10–12 weeks (or at earlier time points if lesion development was excessive), and parasite load in the lesion was determined by limiting dilution analysis [25]. Comparison of parasite load of the different vaccine groups with the vector only control group was performed by ANOVA followed by Dunnett's post hoc test, after normalization of the data.

3. Results

3.1. Immunogenicity of the DNA vaccines

We first evaluated the immunogenicity of the respective DNA vaccines in BALB/c mice immunized twice 2 weeks apart with 100 μg of the different plasmids. Humoral immune response was evaluated 2–3 weeks after the last plasmid DNA injection by measuring total IgG titers by ELISA. Using SLA from the M379 strain as antigen (Fig. 1A), control mice that received saline solution presented very low IgG titers. Mice immunized with the empty plasmid vector VR1012 had slightly higher IgG titers, suggesting some immune stimulation by the plasmid itself. Mice immunized with the different antigen-encoding plasmids showed low but detectable IgG levels, corresponding to 30–40% of that observed in infected animals, indicating that these DNA vaccines induced weak humoral immune responses. There was no major difference in the IgG titers induced by the different DNA vaccines against the M379 *L. mexicana* strain. Similar results were obtained using the Hd18 SLA (Fig. 1B), with the exception of mice immunized with VR1012-LACK, that did not present elevated IgG titers against this *L. mexicana* strain. Also, all IgG titers against the Hd18 strain were about half those against the M379 strain, suggesting a higher background with SLA from strain M379, or possible differences in the immunoreactivity of antibodies elicited by the different DNA vaccines against distinct *L. mexicana* strains.

We also assessed IgG isotypes in the serum of immunized mice. Again, we used SLA from both M379 (not shown) and Hd18 strains (Fig. 2), but found no major differences between both sets of results. DNA vaccines encoding LACK, GP63, and CPb all induced relatively comparable levels of IgG1 and IgG2a, suggesting a rather mixed Th1/Th2 immune response. Only VR1012-GP46 was able to induce significantly higher IgG2a levels suggesting a bias towards a Th1 response.

Cellular immune responses were then evaluated in lymphoproliferation assays after stimulation of spleen cells with SLA from both strains. Stimulation with increasing amount of M379 SLA (from 0.5 to 25 μg/ml, Fig. 3A) had little effect on spleen cells from control mice that received saline solution or the empty plasmid. On the other hand, spleen cells from mice immunized with the antigen-encoding plasmids were increasingly stimulated by the increasing amounts of SLA. These data indicated that all four DNA vaccines could induce a cellular immune response. However, VR1012-LACK induced a somewhat lower proliferative response to M379 SLA, compared to the other vaccines.

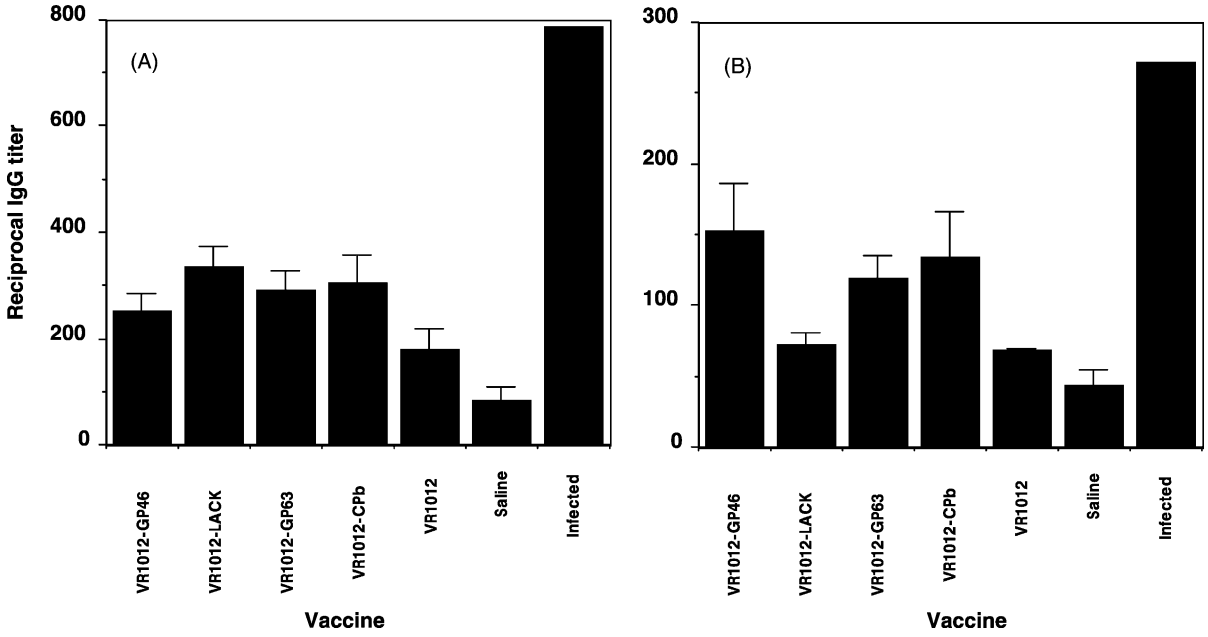

Fig. 1. Serum IgG titers against SLA from strain M379 (A) and Hd18 (B) in response to immunization with distinct DNA vaccines. Mice received two I.M. DNA injections of 100 μg each and IgG titers were determined 2 weeks after the last DNA injection by ELISA using serial dilutions of sera. Mice that received saline solution or that were infected with *Leishmania* (strain Hd18) were used as negative and positive controls, respectively. Results are presented as mean ± S.E.M. of five–nine mice.

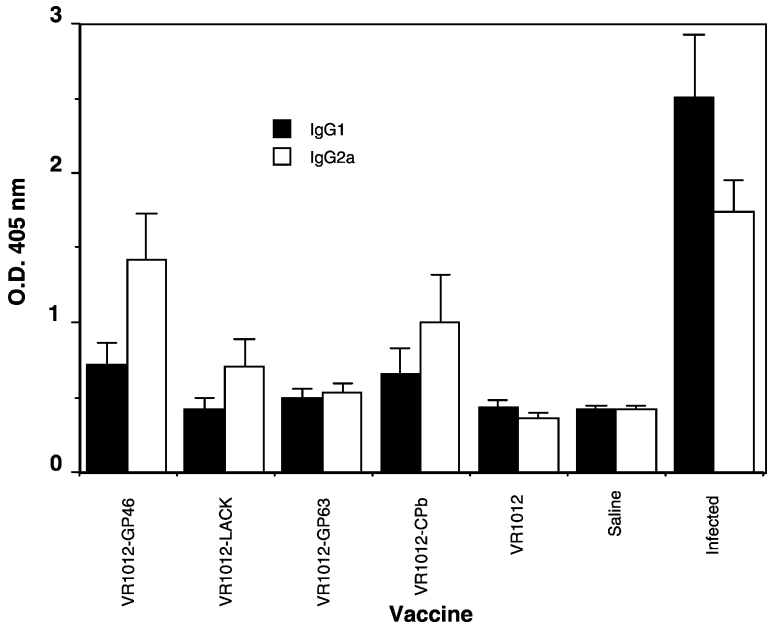

Fig. 2. Serum IgG isotypes levels against Hd18 SLA in response to immunization with distinct DNA vaccines. Mice were immunized as in Fig. 1, and a 1:100 dilution of sera was assayed for IgG1 (dark bars), and IgG2a (light bars). Results are presented as mean ± S.E.M. of five–nine mice.

When stimulated with SLA from the Hd18 strain, there was again a higher proliferation of spleen cells from mice immunized with the antigen-encoding DNA vaccines compared to the control groups (Fig. 3B). However, we did not observe differences between the cellular response induced by the different DNA vaccines. As observed for the humoral response above, cellular immune response may thus vary against distinct strains of *L. mexicana*.

3.2. Efficacy of the DNA vaccines

A key question was then to assess whether the DNA vaccines were able to induce protection against *L. mexicana* infection. Thus, mice immunized with the distinct plasmids were challenged 2–3 weeks after the last DNA injection by the intradermal inoculation of 2×10^6 *L. mexicana* promastigotes in the footpad. We used the strain Hd18 because

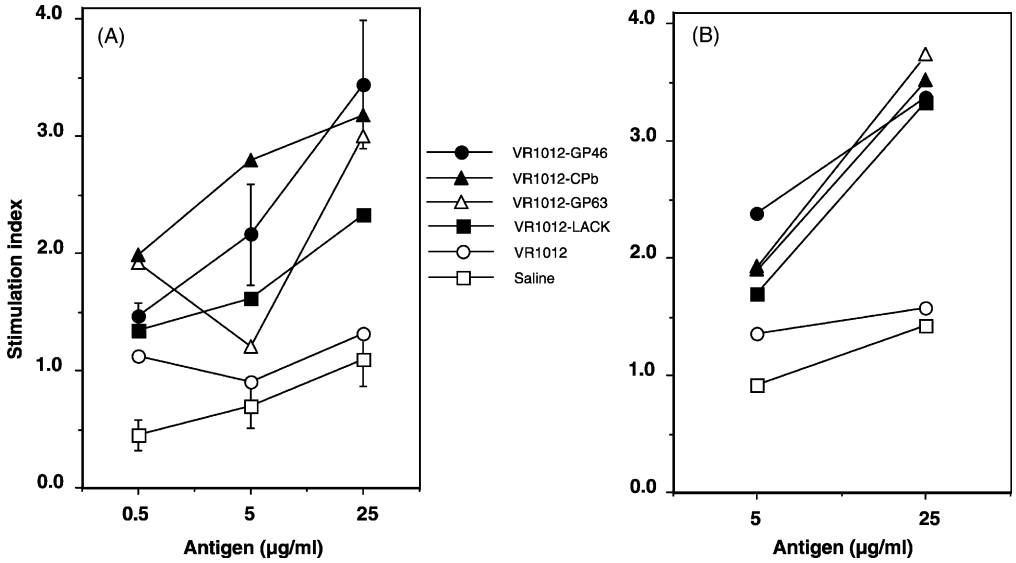

Fig. 3. Proliferative response of single-cell suspension of spleens from mice immunized with different plasmids. Mice were immunized with the indicated plasmids. Spleen cells were stimulated in vitro with various amounts of *L. mexicana* SLA from strains M379 (A) and Hd18 (B), in triplicate wells. The stimulation index was calculated as the ratio of incorporated [^{3}H]thymidine from stimulated/unstimulated cells. Data are presented as the mean of four–six individual mice and for clarity, error bars (S.E.M.) are shown only for the saline and VR1012-GP46 groups (A). The mean of two mice is presented in B.

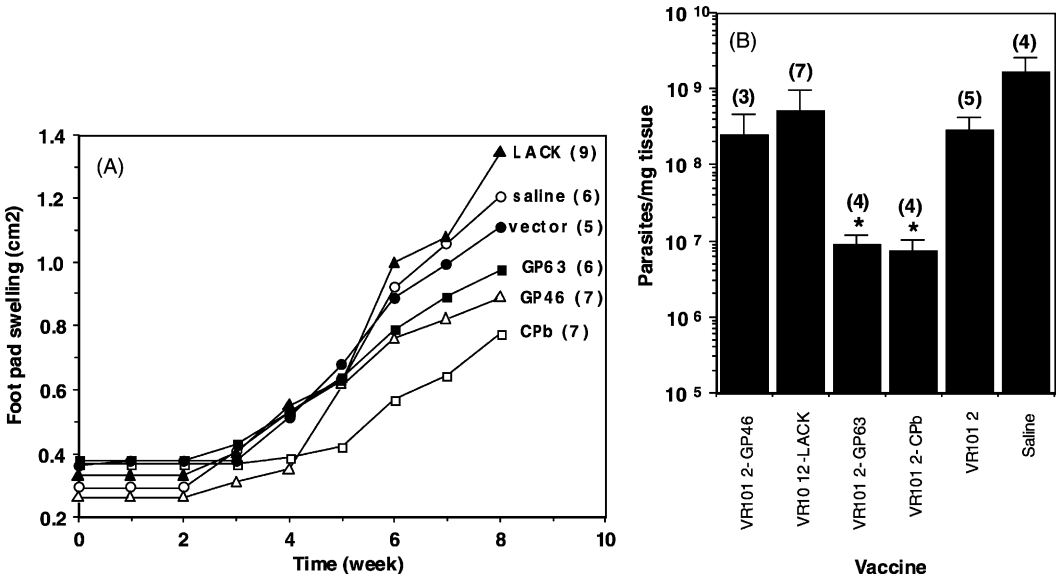

Fig. 4. Infectious challenge of mice immunized with different vaccines. Mice were challenged by the inoculation of 2×10^6 *L. mexicana* parasites (Hd18 strain) in the footpad. Lesion size (A) and parasite burden (B) were measured. Data are presented as the mean ± S.E.M. of the indicated number of mice in brackets, except for lesion size where error bars have been omitted for clarity. The ∗ indicates statistically significant differences in parasite burden compared to the vector only control group ($P < 0.05$).

it was of higher virulence than the strain M379. Lesion development was followed weekly (Fig. 4A), and parasite burden was assessed at the end of the experiments (Fig. 4B). Mice that received saline solution rapidly developed large ulcerating lesions, and some animals had to be euthanized at shorter time points because of the severity of the lesions. High parasite burden in the lesion was also observed in these animals. Mice immunized with the empty vector or VR1012-LACK DNA presented a comparable evolution of the infection, with large lesions and high parasite burden. Mice immunized with VR1012-GP46 presented reduced lesion size, but no significant reduction in parasite burden. On the other hand, mice immunized with VR1012-GP63 and VR1012-CPb had reduced lesion size and significantly lower parasite burden. The best protection was achieved with VR1012-CPb, with over 50% reduction in lesion size and a

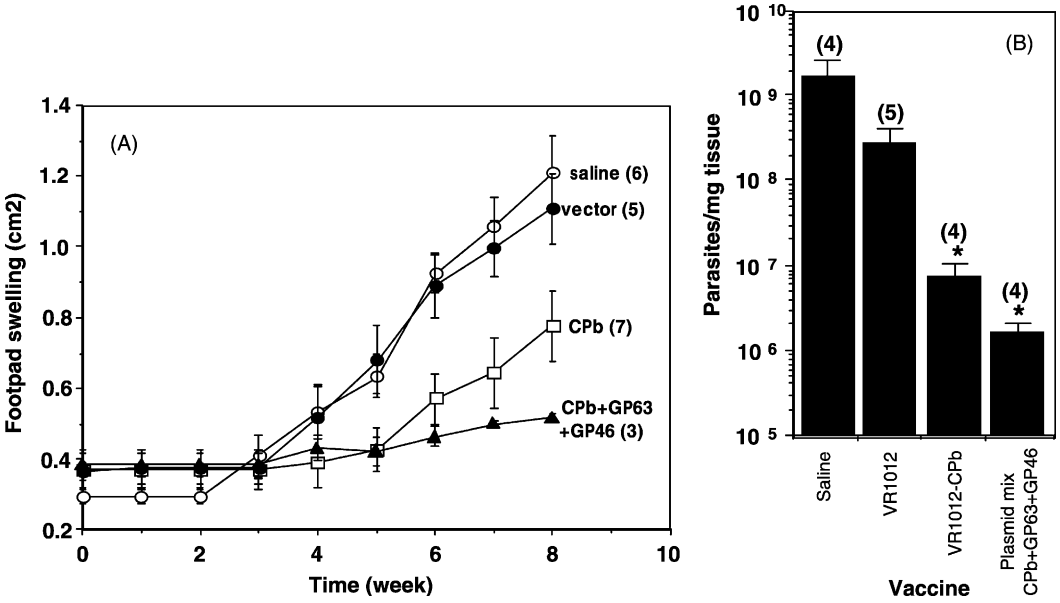

Fig. 5. Infectious challenge of mice immunized with a plasmid DNA mixture. Mice were challenged as in Fig. 4. Lesion size (A) and parasite burden (B) were measured. Data are presented as the mean ± S.E.M. of the indicated number of mice in brackets. The ∗ indicates statistically significant differences in parasite burden compared to the vector only control group ($P < 0.05$).

100-fold lower parasite burden, followed by VR1012-GP63, with reductions of 30% and 100-fold, respectively.

Because protection was still partial, we thus tested whether immunization with a plasmid mixture could enhance protection. We immunized mice twice with 50 μg of each plasmid encoding CPb, GP63, and GP46, and challenged them as before. As shown in Fig. 5, mice immunized with the plasmid mixture developed very small lesions (over 80% reduction, Fig. 5A) and an almost 1000-fold reduction in parasite burden in the lesion (Fig. 5B). Such protection was higher that that obtained with our best single-plasmid vaccine encoding CPb.

4. Discussion

DNA vaccines have been shown to induce a potent and complete immune response particularly suited for the control of intracellular parasite such as *Leishmania*, mostly because of a frequently observed Th1 bias. We thus tested in the present study the immunogenicity and efficacy of DNA vaccines encoding *L. mexicana* antigens GP63, GP46, CPb, and LACK, to determine the usefulness of DNA vaccines, and define the most promising antigens against *L. mexicana*.

4.1. Immunogenicity of the DNA vaccines

The evaluation of humoral and cellular proliferative responses indicated that all four DNA vaccines were able to induce immune responses in BALB/c mice. However, humoral responses were rather weak, in agreement with previous studies showing that a DNA vaccine encoding *L. major* PSA-2 induced barely detectable antibody levels, but significant protection against infection [13]. In fact, the induction of a low humoral response seems to be a general feature of DNA vaccines, which favor cellular rather than humoral responses [26,27]. In addition, part of the humoral immune response we observed was induced by the empty plasmid alone, possibly due to CpG motifs and their adjuvant effect [28,29]. Interestingly, humoral responses against the strain Hd18 where less than half of those against the strain M379, for all vaccines. Even though the humoral response is of little importance against *Leishmania*, this observation suggests that vaccines may be somewhat strain-specific due to significant antigenic differences between strains. In fact, marked polymorphism of antigens such as GP63, considered as a good vaccine candidate against *Leishmania*, has been reported [30,31]. Similar antigen polymorphism has also been observed in other parasite species such as *Plasmodium vivax* [32]. In fact, some of these authors previously stressed the need to take this variability into account for vaccine development [30,31].

The proliferative response observed was of comparable magnitude as that reported after immunization with a mixture of recombinant antigens including CPb and GP63, and various adjuvants [19] suggesting a comparable immunogenicity of DNA and recombinant vaccines. Similarly, our data did not indicate major differences between the immunogenicity of the distinct plasmids. Nonetheless, VR1012-GP46 appeared as a shomewhat better candidate due to a stronger bias towards the production of IgG2a, indicative of a Th1 type immune response.

4.2. Efficacy of the DNA vaccines

We then evaluated the efficacy of these DNA vaccines to induce protection against an experimental infection by *L. mexicana* in susceptible BLAB/c mice. A first interesting observation was that the LACK encoding DNA vaccine did not induce any protection against infection by this species. This is in sharp contrast with the very good protection obtained by a comparable LACK DNA vaccine in the case of *L. major* [15,16,33]. This discrepancy cannot be explained by the absence of cross reactivity between LACK proteins from both species, as they are identical [23]. Rather, it confirms a previous report showing that the LACK antigen does not play a significant role in *L. mexicana* infection as it does in *L. major* infection [23]. This is an important reminder that extrapolation between *Leishmania* species may not be appropriate, and that, as pointed by these authors and others [34,35], the mechanisms of susceptibility/resistance to these parasite species may be different. As a consequence, we may need to design specific vaccines for each species, as cross-protection is still poorly understood and may be elusive [18].

Second, we did obtain increasing levels of protection against infection in mice immunized with DNA vaccines encoding GP46, GP63 and CPb, respectively. This partial protection was observed even though these vaccines appeared to induce a mixed Th1/Th2-type immune response, as indicated by the IgG isotype analysis. Furthermore, a mixture of the three plasmids resulted in better protection than single-plasmid immunization, suggesting potentiation rather than immune interference. Potentiation of protection against *L. major* induced by a cocktail DNA vaccine encoding CPb and CPa has been previously reported [36], whereas no significant effects were observed when combining DNA vaccines encoding TSA and LmSTI1 antigens [37]. Increased immunogenicity, including a stronger Th1 bias, and/or increased protection, rather than immune interference, have also been observed in several studies using mixtures of plasmids encoding different antigens from pathogens such as *Mycobacterium* [38], Bovine Herpesvirus-1 [39], *Plasmodium* [40], or *Schistosoma* [41]. Such flexibility and ease of combining DNA vaccines may thus be a key advantage for vaccine formulation and development, as cocktail vaccines may provide a wider range of potentially protective epitopes, and thus be more likely to overcome possible genetic restriction of the immune response [37].

Good levels of protection against *L. mexicana* have previously been reported, using single or mixture of

recombinant/purified antigens with different adjuvant formulations [19,22], even though only lesion size was assessed in these studies, and not parasite burden. However, as these authors used different strains of mice (C57BL or CBA) and lower parasite doses for the challenge, it is difficult to compare protection levels obtained with these recombinant proteins and our DNA vaccines. It would thus be of key interest to test our DNA vaccines under additional conditions, in particular in other mouse strains.

In conclusion, our study indicates that DNA vaccines are a valuable approach against *L. mexicana*, as they can confer partial protection against an infectious challenge, and this strategy thus warrants further development.

Acknowledgements

The authors wish to thank Dr. Fernando Andrade-Narvaez and his team (Universidad Autónoma de Yucatán, Mérida, Yucatán, Mexico) for their help in the initial stages of this work. We also thank Angel Caamal-Ley for technical assistance. This work was funded by grant T25/181/67 ID# A00220 from the UNDO/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR), and grant 498100-5-J27897M from CONACYT.

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