Immunization with *Leishmania major* Exogenous Antigens Protects Susceptible BALB/c Mice against Challenge Infection with *L. major*

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The potential of *Leishmania major* culture-derived soluble exogenous antigens (SEAgs) to induce a protective response in susceptible BALB/c mice challenged with *L. major* promastigotes was investigated. Groups of BALB/c mice were immunized with *L. major* SEAgs alone, *L. major* SEAgs coadministered with either alum (aluminum hydroxide gel) or recombinant murine interleukin-12 (rmIL-12), *L. major* SEAgs coadministered with both alum and rmIL-12, and *L. major* SEAgs coadministered with Montanide ISA 720. Importantly and surprisingly, the greatest and most consistent protection against challenge with *L. major* was seen in mice immunized with *L. major* SEAgs alone, in the absence of any adjuvant. Mice immunized with *L. major* SEAgs had significantly smaller lesions that at times contained more than 100-fold fewer parasites. When lymphoid cells from *L. major* SEAg-immunized mice were stimulated with leishmanial antigen in vitro, they proliferated and secreted a mixed profile of type 1 and type 2 cytokines. Finally, analyses with Western blot analyses and antibodies against three surface-expressed and secreted molecules of *L. major* (lipophosphoglycan, gp46/M2/PSA-2, and gp63) revealed that two of these molecules are present in *L. major* SEAgs, lipophosphoglycan and the molecules that associate with it and gp46/M2/PSA-2.

*Leishmania major* is an etiological agent of cutaneous leishmaniasis, a disease characterized by cutaneous lesions that can be self-resolving with life-long immunity or chronic when accompanied by defective cellular immune responses (24). The disease is prevalent in many tropical and subtropical regions of the world, where it is transmitted via the bite of an infected sand fly.

To date, there are no proven vaccines against any form of leishmaniasis; however, several approaches are being tested (reviewed in references 6, 8, 18, and 23). In brief, vaccines based on killed promastigotes with or without *Mycobacterium bovis* BCG have shown significant protection against *L. braziliensis, L. mexicana*, and *L. donovani* in humans. In addition, in experimental animal models, several other vaccine preparations are being tested. Examples include attenuated live parasites; subunit vaccines delivered by live carriers such as *M. bovis* BCG expressing the surface protease gp63 of *L. major*; vaccinia virus expressing the glycoprotein gp46/M2/PSA-2; gp63 expressed in attenuated *Salmonella enterica serovar Typhimurium*; purified recombinant or native proteins formulated with an adjuvant such as the *Leishmania* homologue of receptors for activated C kinase plus interleukin-12 (IL-12), gp46/M2/PSA-2, or protein dp72 plus *Corynebacterium parvum*, T-cell epitopes plus Poloxamer 407; and vaccination with DNA encoding gp63, leishmanial activated C kinase, or gp46/M2/PSA-2. In general, these vaccination protocols elicited partial protection against *L. major*.

Protection of mice against *L. major* infection depends on the ability to generate macrophage-activating Th1 responses resulting in the production of gamma interferon (IFN-γ) and low levels of IL-4 (28). Furthermore, there is considerable evidence that Th2-type responses and the production of IL-4 result in the inability to control disease or in disease exacerbation (9, 10). However, this Th1/Th2 paradigm is currently being expanded. New evidence suggests that IL-10 is an additional factor controlling susceptibility to *L. major* in BALB/c mice (21). In addition, the idea that IL-4 might act in concert with IL-13 to produce an additive effect on *L. major* susceptibility has also been reported (17). All these reports suggest that a cumulative effect of three disease-promoting cytokines, IL-4, IL-10, and IL-13, may be important to disease outcome.

The present study was aimed at evaluating the potential of soluble culture-derived *Leishmania* secreted and excreted exogenous antigens (*L. major* SEAgs) as vaccine candidates against *L. major* infections in the susceptible BALB/c mouse model. Recently, these exoantigens were used to develop serological assays that demonstrated very high degrees of sensitivity and specificity. These assays efficiently detected antileishmanial antibodies, both immunoglobulin M (IgM) and IgG, in visceral leishmaniasis patients (15, 25). These findings, supported by Western blot patient profiles, indicated that leishmanial exoantigens are excellent diagnostic markers and are highly immunogenic in the infected host. In addition, recent work by Webb et al. (30) showed that culture filtrate proteins of *L. major* promastigotes (produced with a different strain of

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parasite with different production techniques) administered to mice with *Corynebacterium parvum* protected against a subsequent challenge with parasites.

The adjuvant administered with an antigen influences the quantity and quality of the ensuing immune response to the antigen. Therefore, we injected BALB/c mice with *L. major* SEAGs alone or in conjunction with adjuvants currently approved or close to approval for use in humans and formulations that have shown promise in vaccine trials. Aluminum in the form of aluminum hydroxide, aluminum phosphate, or alum has been commonly used as an adjuvant in many vaccines licensed by the U.S. Food and Drug Administration (3). IL-12 is a critical component in the development of cell-mediated immunity and stimulates cell proliferation and IFN-γ secretion by T and natural killer cells (11). Importantly, IL-12 has the ability to promote the development of CD4 Th1 cells, which are necessary for protective immunity in leishmaniasis (11, 29).

It has also been shown that adsorption of both antigen and IL-12 onto alum enhances immunostimulatory effects that promote both humoral and Th1 cytokine responses to human immunodeficiency virus infection in mice (12) and to *L. major* in rhesus monkeys (13). Montanide ISA 720, a metabolizable oil-based adjuvant, has also been tested in humans (2).

We demonstrate here that, unlike most *Leishmania* antigens, which have shown protection only when administered with adjuvants, *L. major* SEAGs are unusual in that a single vaccination with these antigens alone resulted in significant inhibition of lesion development in susceptible BALB/c mice following challenge infection with *L. major*.

**MATERIALS AND METHODS**

*L. major* parasites. Metacyclic promastigotes of *L. major* strain LV 39 (RHO-SU-59-P) were used. Parasites were maintained as previously described (27), and metacyclics were isolated from stationary-phase cultures by negative selection for IL-4 and IFN-γ with commercial anticytokine antibody pairs (Becton Dickinson/Pharmingen, San Jose, Calif.) and protocols provided by the manufacturer. Alternatively, after 5 days, the cultures were pulsed with 1 μCi of [3H]hypoxanthine (5 Ci/mmol; Amershams, Arlington Heights, Ill.) for 18 h and harvested, and cell proliferation was evaluated by liquid scintillation counting.

Preparation of parasite extracts. Stationary-phase *L. major* promastigotes were collected, centrifuged, washed three times, and resuspended in phosphate-buffered saline (PBS) to a final concentration of 5 × 10⁶ parasites/ml in the presence of protease inhibitor cocktail set III (Calbiochem, San Diego, Calif.) and frozen at −20°C. The protease inhibitor cocktail contained AEBSF hydrochloride (2 mM final concentration), aprotinin (1.6 μM), bestatin (100 μM), E-64 (30 μM), leupeptin hemisulfate (40 μM), and pepstatin A (20 μM). The parasites were subjected to three cycles of freezing and thawing and centrifuged at 13,800 × g at 4°C for 10 min. The pellets were then extracted with Triton X-100 (1% in PBS) with protease inhibitors and centrifuged again at 13,800 × g at 4°C for 10 min to collect the Triton X-100 fractions that were used in the Western blot analyses.

SDS-PAGE. To obtain a silver-stained profile of the main constituents of the *L. major* SEAGs, 200 μl of the preparation (1,292 μg/ml) was precipitated with 3 volumes of cold acetone and kept at −20°C for 24 h. The precipitate was collected by centrifugation at 13,800 × g at 4°C for 10 min, dried, and left to dry. The precipitate was then resuspended in 20 μl of PBS and mixed with 3 μl of 0.5 M dithiothreitol and 7.5 μl of 4× LDS buffer (Invitrogen, Carlsbad, Calif.). The mixture was incubated at 90°C for 5 min on ice before the electrophoresis; 10 μl of the mixture was loaded into a precast sodium dodecyl sulfate (SDS)–4 to 12% polyacrylamide gel electrophoresis (PAGE) gel (Invitrogen) and run in morpholineethanesulfonic acid (MES) buffer at 200 V for 35 min. The gel was fixed and silver stained following the recommendations of the manufacturer of silverXpress (Invitrogen). As molecular weight standards, a prestained protein mix (Amershams Biosciences, Piscataway, N.J.) was used.

Western blot analysis. The proteins of the *L. major* SEAGs and the Triton X-100 extract of *L. major* promastigotes were electrophoretically separated as indicated above and then transferred to nitrocellulose paper (Bio-Rad, Hercules, Calif.) in Tris-HCl–glycine–methanol buffer at 100 V for 1 h. The membrane was blocked with 1% milk in PBS for 1 h and probed with the following antibodies against *Leishmania* components for 1 h at room temperature: mouse monoclonal antibody 235 against gp63 (gift of R. McMaster, University British Columbia, Vancouver, Canada) diluted 1:1,000 in PBS; mouse monoclonal antibody 79.3 against lipophosphoglycan (gift of S. Turco, University of Kentucky, Lexington) diluted 1:1,000; and rabbit polyclonal anti-gp46 (gift of D. McMahon-Pratt, Yale University, New Haven, Conn.) diluted 1:100. Controls consisted of normal mouse and normal rabbit sera. The membrane strips were then washed three times with PBS containing 0.2% Tween 20 (PBS-T) and incubated with a 1:1,000 dilution of goat antibodies specific for mouse IgG (heavy and light chains) or rabbit IgG (heavy and light chains) conjugated to alkaline phosphatase (KPL, Gaithersburg, Md.). After 1 h, the membranes were washed three times with PBS-T and then incubated with the phosphatase substrate nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) (KPL) for 15 min at room temperature in the dark.
**RESULTS**

**L. major** **SEAgs** prime specific cells in susceptible BALB/c mice. Our first goal was to assess the immunogenicity of **L. major** **SEAgs** in **L. major**-susceptible BALB/c mice. To achieve this, BALB/c mice were injected subcutaneously with 50 μg of **L. major** **SEAgs** in the rump. Ten days later, immunized mice were collected and pooled for lymphocyte proliferation assays. Mononuclear cell suspensions adjusted to 2 × 10^6/ml were prepared as described in Materials and Methods. These cells were stimulated in vitro with 2 × 10^5 **L. major**/ml or 50 μg of **L. major** **SEAgs/ml**. After 5 days, the cultures were assessed for proliferation as described in Materials and Methods.

**L. major** **SEAgs** stimulate protective immunity against a subsequent challenge with **L. major** in susceptible BALB/c mice. Our second goal was to assess the ability of **L. major** **SEAgs** to protect against a subsequent challenge with **L. major** in susceptible BALB/c mice. BALB/c mice were injected subcutaneously with 50 μg of **L. major** **SEAgs**, and 10 days later the immunized mice together with controls were challenged with 10^5 metacyclic LV 39 promastigotes subcutaneously in one hind footpad. Lesion sizes in mice were monitored by measuring the increase in size of the infected footpad. As shown in Fig. 2, one injection of 50 μg of **L. major** **SEAgs** alone without adjuvant induced significant protection (*P* < 0.05) against a subsequent challenge with **L. major**. This finding was corroborated by lesion parasite burden analyses (Table 1).

**Vaccination with **L. major** **SEAgs** plus rmIL-12 in alum also proved to be protective but no more protective than **L. major** **SEAgs** alone.** Protection with **L. major** **SEAgs** alone prompted us to evaluate the effects of coinjecting these antigens with adjuvants. We tested the conventional adjuvants alum, rmIL-12, and Montanide ISA 720. Alum is currently the only Food and Drug Administration-approved adjuvant in clinical use in humans. The other adjuvants, rmIL-12 and Montanide ISA 720, have shown good results in several primate and human vaccination studies.

The results showed that coinjecting **L. major** **SEAgs** together

**TABLE 1. Parasite burden in lesions of BALB/c mice immunized once with **SEAgs****

<table>
<thead>
<tr>
<th>Expt</th>
<th>No. of <strong>L. major</strong> (10^5)/footpad (95% confidence limits)</th>
<th>Reduction in parasite burden (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Vaccinated</td>
</tr>
<tr>
<td>I</td>
<td>9.18 (5.04–13.33)</td>
<td>0.83 (0.41–1.25)</td>
</tr>
<tr>
<td>II</td>
<td>5.30 (1.60–8.90)</td>
<td>0.20 (0.03–0.30)</td>
</tr>
<tr>
<td>III</td>
<td>7.25 (2.38–9.70)</td>
<td>0.21 (0.04–0.38)</td>
</tr>
</tbody>
</table>

* Mice were treated as described in the legend to Fig. 2. Twenty-eight days postinfection, duplicate mice were killed, and the parasite burdens in their footpads were analyzed with the limiting dilution assay described in Materials and Methods. The table presents three replicate experiments.
with alum, rmIL-12, or Montanide ISA 720 did not increase protection. The only adjuvant system that equaled the protection seen with L. major SEAgs alone was IL-12 plus alum (Fig. 3). This conclusion was corroborated by the fact that similar numbers of parasites were present in the lesions of mice treated with L. major SEAgs alone or L. major SEAgs plus IL-12 plus alum (Table 2). However, interestingly, immunization with L. major SEAgs or L. major SEAgs plus IL-12 plus alum led to the induction of dissimilar levels of the Th1-associated cytokine IFN-γ, with L. major SEAgs plus IL-12 plus alum-treated mice producing approximately 10-fold less IFN-γ when cells from the mice were restimulated with L. major parasites in vitro (Table 3).

It should be mentioned that we also immunized mice twice with either L. major SEAgs plus alum or plus IL-12 or plus alum, rmIL-12, or Montanide ISA 720. Ten days later, mice were challenged in the left hind footpad with 10^7 L. major metacyclic promastigotes. At 28 days postinfection, duplicate mice were killed, and the parasite burdens in their footpads were analyzed with the limiting dilution assay described in Materials and Methods.

**TABLE 2.** Parasite burden in lesions of BALB/c mice vaccinated with SEAgs with and without adjuvants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of L. major (10^6)/footpad (95% confidence limits)</th>
<th>Reduction in parasite burden (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>5.3 (1.6–8.9)</td>
<td></td>
</tr>
<tr>
<td>SEAgs + 10 μg of alum</td>
<td>2.9 (0.1–0.5)</td>
<td>1.8</td>
</tr>
<tr>
<td>SEAgs + rmIL-12</td>
<td>5.0 (0.1–0.8)</td>
<td>1.1</td>
</tr>
<tr>
<td>SEAgs + Montanide ISA 720</td>
<td>3.3 (0.7–5.8)</td>
<td>1.6</td>
</tr>
<tr>
<td>SEAgs + rmIL-12 + alum</td>
<td>0.2 (0.1–0.2)</td>
<td>26.5</td>
</tr>
<tr>
<td>SEAgs alone</td>
<td>0.2 (0.04–0.4)</td>
<td>25.3</td>
</tr>
</tbody>
</table>

a BALB/c mice were immunized with 50 μg of SEAgs alone or coadsorbed with alum, rmIL-12, rmIL-12 coadsorbed in alum, or Montanide ISA 720. Ten days later, mice were challenged in the left hind footpad with 10^7 L. major metacyclic promastigotes. At 28 days postinfection, duplicate mice were killed, and the parasite burdens in their footpads were analyzed with the limiting dilution assay described in Materials and Methods.

Montanide ISA 720 and then challenged the mice with L. major. This engendered low levels of protection (data not shown) similar to the levels of protection seen in Fig. 3. We therefore concluded that the only two systems worth exploring further were immunization with L. major SEAgs alone and immunization with L. major SEAgs plus IL-12 plus alum.

Double immunizations with the L. major SEAgs alone proved to be more protective than double immunizations with L. major SEAgs plus IL-12 plus alum. Groups of BALB/c mice were injected subcutaneously with L. major SEAgs alone or L. major SEAgs plus IL-12 plus alum and 13 days later (7) subcutaneously again with the same preparations. Thirteen days after the second immunization, duplicate vaccinated and control mice were sacrificed, and their spleen cells were collected and pooled for lymphocyte proliferation assays. Mononuclear cells were stimulated in vitro with L. major, L. major SEAgs, or medium alone. Five days later, the degree of proliferation of the cells was assessed as described in Materials and Methods. The results showed that mononuclear cells harvested from mice immunized twice with L. major SEAgs or L. major SEAgs plus rmIL-12 plus alum showed enhanced proliferative responses compared to those from mice given a single immunization with L. major SEAgs (compare Fig. 1 and 4).

Thirteen days after the second immunization, immunized mice together with their controls were challenged with L. major in a hind footpad. Immunizing twice with L. major SEAgs if anything was more protective than immunizing once (compare Fig. 2 and 5); e.g., after a single immunization, lesion size was approximately 1 mm at 28 days postinfection, whereas after two immunizations, lesions were almost nonexistent at 28 days. Curiously, in sharp contrast to immunizing twice with L. major SEAgs alone, immunizing twice with L. major SEAgs plus IL-12 plus alum induced little if any protection (Fig. 5). The same result was seen when the parasite burden within the lesions was assessed; the only group that showed a substantial decrease in parasite burden was the group immunized with L. major SEAgs alone (Table 4), a 53-fold reduction at day 28 and a 125-fold reduction at day 38 postinfection.

**TABLE 3.** Cytokines produced by BALB/c mice vaccinated with SEAgs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stimulus</th>
<th>Cytokine concn (pg/ml ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>L. major</td>
<td>62 ± 0 19.1 ± 1.8</td>
</tr>
<tr>
<td>SEAgs</td>
<td>L. major</td>
<td>0 ± 0 7.6 ± 0.6</td>
</tr>
<tr>
<td>SEAgs alone</td>
<td>L. major</td>
<td>683 ± 52 1.2 ± 0.9</td>
</tr>
<tr>
<td>SEAgs + rmIL-12 + alum</td>
<td>L. major</td>
<td>828 ± 52 10.2 ± 0.9</td>
</tr>
<tr>
<td>SEAgs + rmIL-12 + alum</td>
<td>L. major</td>
<td>42 ± 1 1.3 ± 0.1</td>
</tr>
<tr>
<td>SEAgs + Montanide ISA 720</td>
<td>L. major</td>
<td>442 ± 11 9.0 ± 1.7</td>
</tr>
<tr>
<td>SEAgs + Montanide ISA 720</td>
<td>L. major</td>
<td>442 ± 11 9.0 ± 1.7</td>
</tr>
</tbody>
</table>

a Mice were vaccinated as described in the legend to Fig. 2. Cells were harvested from the draining lymph nodes (inguinal and popliteal) 7 days postinfection. Cells were incubated at 37°C with 50 μg of SEAgs or 2 x 10^6 L. major/ml. Culture supernatants were collected 48 h later, and the concentrations of IFN-γ and IL-4 were determined by ELISA as described in Materials and Methods.

Vaccination with L. major SEAgs induces a mixed Th1/Th2-type cytokine response. In order to dissect the nature of the immune response induced by immunization with either L. major SEAgs alone or L. major SEAgs plus IL-12 plus alum, we
infection.

Differences between lesion sizes in control mice versus mice immunized with L. major SEAg plus rmIL-12 plus alum produced more cytokines (IFN-γ, IL-4, and IL-10) than cells from mice immunized with L. major SEAg alone (Fig. 6). This was true whether the spleen cells from immunized mice were restimulated in vitro with L. major SEAg or with L. major parasites (Fig. 6). Therefore, immunization with L. major SEAg induced a mixed Th1/Th2-type cytokine response.

Next, we determined the cytokines produced after immunized mice were challenged with L. major. Twenty-eight days after immunized mice were challenged (a time when mice immunized with L. major SEAg alone were resisting infection but mice immunized with L. major SEAg plus rmIL-12 plus alum were not resisting infection; Fig. 5), cells from the mice were again restimulated in vitro with either L. major SEAg or L. major parasites. As can be seen in Fig. 7, mice immunized with L. major SEAg alone produced IFN-γ but low or undetectable levels of IL-4 and IL-10. In contrast, mice immunized with L. major SEAg plus rmIL-12 plus alum in general produced much larger amounts of IL-4 and IL-10 and did not produce IFN-γ when restimulated in vitro with L. major parasites. Thus, at this time of infection, mice vaccinated with L. major SEAg alone produced more type 1 cytokines, while mice vaccinated with L. major SEAg plus rmIL-12 plus alum produced more type 2 cytokines (Fig. 7).

L. major SEAg contain at least the leishmanial surface antigens lipophosphoglycan and gp46. In an effort to characterize L. major SEAg (since exo antigens of L. donovani and L. mexicana have been described before [15, 25] but not those of L. major), we silver-stained SDS-PAGE gels of the antigens and probed Western blots of the antigens with antibodies against common surface components of L. major. These analyses revealed that the L. major SEAg contained approximately 10 to 12 major components by SDS-PAGE analysis (Fig. 8A) and that among these are the surface molecules lipophosphoglycan and gp46 (Fig. 8B). On the other hand, gp63 was not detectable in the L. major SEAg (Fig. 8B).

**DISCUSSION**

In the present study, we investigated the potential of L. major culture-derived exoantigens (L. major SEAg) alone and in conjunction with various adjuvants to elicit a protective
immune response against challenge with the parasite in susceptible BALB/c mice. Recently, leishmanial exoantigens were used to develop ELISAs, which have very high sensitivity and specificity for antileishmanial antibodies (both IgM and IgG) in patients and in dogs infected with visceral leishmaniasis (15, 25). These findings suggested that exoantigens are highly immunogenic in these two hosts and thus might be good vaccine candidates.

We found that *L. major* SEAg stimulated cells in BALB/c mice injected with the antigens and that *L. major* SEAg also induced a recall response, since cells taken from mice injected twice with *L. major* SEAg proliferated more in vitro when restimulated with *L. major* SEAg than cells taken from mice injected only once with *L. major* SEAg (compare Fig. 1 and 4). Moreover, a single injection of *L. major* SEAg (in the absence of adjuvant) induced significant resistance to a subsequent challenge with *L. major*, with respect to both lesion size (Fig. 2) and parasite burden (Table 1).

The fact that no adjuvant was required for *L. major* SEAg to induce protection was not expected, but this showed that *L. major* SEAg are highly immunogenic in mice, as they have been shown to be in humans and dogs (15, 25). Since *L. major* SEAg contain substantial amounts of lipophosphoglycan (Fig. 8) and lipophosphoglycan has been shown to have adjuvant-like activities (19, 31) which lead to enhanced cell proliferation and cytokine production, it is tempting to speculate that the immunogenicity of *L. major* SEAg is due to their lipophosphoglycan content (Fig. 8). Thus, lipophosphoglycan, and perhaps other components of *L. major* SEAg that have adjuvant qualities, may be responsible for the ability of *L. major* SEAg to stimulate protection against *L. major* challenge in the absence of adjuvant.

Although stimulating mice once with either *L. major* SEAg alone or *L. major* SEAg in conjunction with IL-12 and alum...
The only adjuvant that induced protection with *L. major* SEAgS led to similar degrees of protection (Fig. 3), stimulating mice twice with either of these preparations led to different outcomes of infection. Double injections of *L. major* SEAgS alone induced greater protection against challenge with *L. major*, but unexpectedly, double injections of *L. major* SEAgS plus IL-12 plus alum induced no protection (Fig. 5).

In an effort to define the mechanism underlying the inability of double injections of *L. major* SEAgS plus IL-12 plus alum to induce protection against *L. major* challenge in BALB/c mice, we analyzed the cytokines produced by mice treated with either *L. major* SEAgS alone or *L. major* SEAgS plus IL-12 plus alum. All cytokine data are contained in Table 3 and Fig. 6 and 7. The only clear difference in Table 3 (mice immunized once with either *L. major* SEAgS or *L. major* SEAgS plus IL-12 plus alum) is that the antigen plus IL-12 plus alum-treated mice produced less IFN-γ, especially when challenged with *L. major* parasites in vitro. Thus, even after a single injection of *L. major* SEAgS plus IL-12 plus alum, mice were less able to produce IFN-γ; however, this amount of IFN-γ must have been sufficient, since the mice were protected from infection.

When cells from mice injected twice with *L. major* SEAgS plus IL-12 plus alum were harvested before the animals were infected with *L. major* parasites (Fig. 6), the cells produced more of all cytokines in vitro, but especially IFN-γ and IL-10. When cells were harvested from the mice after infection with *L. major* (Fig. 7), they continued to produce more IL-10 whether they were stimulated with *L. major* SEAgS or with *L. major* parasites. In contrast, cells from *L. major* SEAgS plus IL-12 plus alum-treated mice no longer produced more IFN-γ when stimulated with *L. major* SEAgS, and the cells produced nearly undetectable levels of IFN-γ when they were stimulated with *L. major* parasites (a result similar to that shown in Table 3).

Therefore, cells from mice vaccinated twice with *L. major* SEAgS plus IL-12 plus alum tended to produce less IFN-γ (especially when the cells were harvested from infected mice and the cells were restimulated in vitro with *L. major* parasites), but more IL-10. IL-10 is known to favor the survival of *L. major* in mice. Indeed, it has been shown by Sacks and colleagues (4, 5) that resistant mice harbor a low number of parasites in their tissues for their lifetime unless IL-10 is neutralized in the mice, which leads to complete destruction of *L. major*. That is, survival of *L. major* in mice is determined by the interplay that occurs between IFN-γ (which induces parasite destruction) and IL-10 (which favors parasite survival). If one or the other cytokine is overproduced, the parasite will or will not be destroyed, respectively.

IL-12 has been shown to induce both IFN-γ and IL-10 in mice injected with the cytokine (20). Thus, it is possible that in mice treated with *L. major* SEAgS plus IL-12 plus alum, the IL-12 present in the inoculum induced high levels of not only IFN-γ but also IL-10. In fact, this was the case, since mice treated with *L. major* SEAgS plus IL-12 plus alum were producing high levels of both IFN-γ (although cells from these animals did not produce IFN-γ when restimulated with *L. major* in vitro; Fig. 7) and IL-10 prior to infection with *L. major* (Fig. 6) and after infection (Fig. 7) with the parasite. In contrast, mice vaccinated with *L. major* SEAgS alone produced less IL-10 both before and after infection (in some cases as much as 10-fold less; Fig. 6 and 7). Thus, the ability of double injections of *L. major* SEAgS plus IL-12 plus alum to stimulate IL-10 production (Fig. 6 and 7) may be the reason it was unable to induce protection in treated mice.

It is important to mention that Noormohammadi et al. (22) recently made observations that may have direct bearing on the results presented here. These authors were studying the effect of including IL-12 DNA on the immunogenicity of gp46/M2/PSA-2 DNA for mice. While gp46/M2/PSA-2 DNA alone induced protection against a subsequent challenge with *L. major*, co-administering gp46/M2/PSA-2 and IL-12 DNA abolished protection. Since gp46/M2/PSA-2 is a component of *L. major* SEAgS (Fig. 8), it is possible that these authors observed a phenomenon similar to that reported here.

Taken as a whole, the results presented here confirm that *L. major* SEAgS are highly immunogenic and that they can elicit significant protection against challenge with *L. major* in susceptible BALB/c mice. Indeed, Webb et al. reported that injecting mice with leishmanial culture filtrate proteins plus *Corynebacterium parvum* also induced a mixed type 1-type 2 response and resistance to infection with *L. major* (30). Thus, a different form of the excreted and secreted antigens of *Leishmania* spp. also appears to be immunogenic and capable of inducing protection against challenge with *L. major*. Perhaps the most interesting attribute of *L. major* SEAgS is their ability to elicit protection in the absence of any adjuvant. Therefore, *L. major* SEAgS may be useful not only as a diagnostic tool but...
also in the development of vaccines against Old World cutaneous leishmaniasis.

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