CROSS-PROTECTION AGAINST \textit{LEISHMANIA DONOVANI} BUT NOT \textit{L. BRAZILIENSIS} CAUSED BY VACCINATION WITH \textit{L. MAJOR} SOLUBLE PROMASTIGOTE EXOGENOUS ANTIGENS IN BALB/c MICE

WILLY K. TONUI AND RICHARD G. TITUS*
Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya; Department of Microbiology Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado

Abstract. Vaccinating with soluble \textit{Leishmania major} promastigote exogenous antigens (\textit{LmSEAg}s) protects mice against challenge with \textit{L. major}. To explore the potential of \textit{LmSEAg}s to cross-protect against infection with other species of \textit{Leishmania}, BALB/c mice were immunized with \textit{LmSEAg}s prior to challenge with either \textit{L. donovani} or \textit{L. braziliensis} promastigotes. Such mice were protected against \textit{L. donovani} but not \textit{L. braziliensis} infection.\textit{Leishmania braziliensis}-infected mice developed lesions that were not significantly different from those of controls and that contained 13-fold more parasites. In contrast, immunized mice infected with \textit{L. donovani} were protected as illustrated by low splenic parasite loads (as much as 4,913-fold fewer parasites). This protection corresponded to significant increases in gamma interferon and low production of interleukin-4 (IL-4) IL-4 or IL-10, which suggested an enhanced type 1 response.

INTRODUCTION

Parasites of the genus \textit{Leishmania} are transmitted by the bite of sand flies and infect cells of the mononuclear phagocyte lineage of their vertebrate hosts.\textsuperscript{1} Depending on the virulence factors of the parasite and the immune response established by the host, a spectrum of illnesses known as leishmaniasis can appear, and these can be cutaneous and/or visceral. The World Health Organization estimates that at least 350 million people are at risk worldwide, 12 million people are infected with \textit{Leishmania} parasites, and that as many as two million new cases of leishmaniasis occur each year throughout Africa, Asia, Europe, and the Americas.\textsuperscript{3} With the advent of the human immunodeficiency virus epidemic, leishmaniasis has surged as a reactivating infection in patients with acquired immunodeficiency syndrome in many parts of the world.\textsuperscript{2,4}

\textit{Leishmania braziliensis} is an intracellular protozoan parasite that infects persons living in Central and South America. Mucocutaneous leishmaniasis, which is caused by infection with \textit{L. braziliensis}, begins with skin ulcers, which spread and cause dreadful and massive tissue destruction, especially of the nose and mouth.\textsuperscript{5} \textit{Leishmania donovani}, which causes visceral leishmaniasis (kala-azar), is the most severe form of leishmaniasis; it involves the internal organs and can be fatal if left untreated.\textsuperscript{4} It is characterized by irregular fever, weight loss, hepatosplenomegaly, and anemia.

Although the immunology of visceral leishmaniasis has received considerable attention, efforts toward vaccinating against leishmaniasis have focused principally on localized cutaneous disease.\textsuperscript{5} An important consideration in vaccination is protection against challenge by heterologous strains or species of parasites.\textsuperscript{6,7}

\textit{Leishmania major} promastigote exogenous antigens (\textit{LmSEAg}s) have been shown to protect against \textit{L. major} infection in BALB/c mice.\textsuperscript{8} These exo-antigens have also been used to develop serologic assays that efficiently detected antibodies to \textit{Leishmania} (both IgM and IgG) in visceral leishmaniasis patients.\textsuperscript{9,10} In the present study, we tested the efficacy of immunization with \textit{LmSEAg}s to elicit protective immunity in BALB/c mice against \textit{L. donovani} or \textit{L. braziliensis}. Our studies indicate that mice immunized with \textit{LmSEAg}s were protected against infection with \textit{L. donovani} but not against infection with \textit{L. braziliensis}.

MATERIALS AND METHODS

Mice and parasites. Young, adult, female BALB/c mice obtained from the National Cancer Institute (Bethesda, MD) were used in all experiments. These experiments complied with all relevant federal guidelines and institutional policies. Stationery phase promastigotes of \textit{L. braziliensis} strain LTB 111 and \textit{L. donovani} strain 2S were used. Parasites were maintained as previously described.\textsuperscript{11}

Preparation of \textit{LmSEAg}s and immunization of BALB/c mice. The \textit{LmSEAg}s were produced as previously described by Ryan and others\textsuperscript{9} and Martin and others.\textsuperscript{10} Mice were immunized by subcutaneous injections of 50 \( \mu \)g of \textit{LmSEAg}s in the absence of any adjuvant.\textsuperscript{8} Controls received vehicle alone. Mice were given booster immunizations with \textit{LmSEAg}s or vehicle 13 days after the initial immunization.\textsuperscript{8} In previous experiments with \textit{LmSEAg}s, we used various adjuvants (e.g., alum, interleukin-12 [IL-12]) and these did not enhance the protective effect of \textit{LmSEAg}s. The same observations were made in the studies presented here; thus, all results in this report were achieved by the injection of \textit{LmSEAg}s alone.

Infection of mice and determination of parasite numbers in cutaneous lesions or spleens. Thirteen days after the animals were given booster immunizations, some of the mice were challenged subcutaneously in the left hind footpad with 1 \( \times \) \( 10^7 \) \textit{L. braziliensis}. Lesion development was then followed by measuring the thickness of the infected footpad and compared with the thickness of the same footpad prior to infection. At various times, two mice were killed to determine the parasite burdens in the footpads using a published limiting dilution assay.\textsuperscript{12}

Other groups of mice were infected intravenously through the lateral tail vein with 1 \( \times \) \( 10^6 \) \textit{L. donovani}.\textsuperscript{13} At various
times, two mice were killed to determine spleen weight and parasite burdens in the spleens.12

**Cell proliferation and cytokine responses of cells from mice immunized with LmSEAg**s. Thirteen days after the booster immunization with LmSEAg, spleen cells were harvested from control (injected with the vehicle used to inject LmSEAg) or immunized mice. Splenic mononuclear cells were isolated using Ficoll gradients (Sigma, St. Louis, MO)8. Cells were adjusted to a concentration of 2 × 10⁶/mL in Dulbecco’s modified Eagle’s medium containing 0.5% normal mouse serum (Harlan Bioproducts, Indianapolis, IN) and 5 × 10⁻⁵ M β-mercaptoethanol (Sigma). Cells were stimulated with 50 μg/ml of LmSEAg, 2 × 10⁶/mL of L. donovani or L. braziliensis, or medium alone (negative control). Supernatants were collected after 48 hours of culture (a time we found to be optimal for the cytokines measured) and tested using standard sandwich enzyme-linked immunosorbent assays for IL-4, interferon-γ (IFN-γ), and IL-10 using commercial anti-cytokine antibody pairs (Becton Dickinson/Pharmingen, San Jose, CA) and protocols provided by the manufacturer. Alternatively, after five days, the cultures were pulsed with 1 μCi of ³H-thymidine (5 Ci/mmol; Amersham, Arlington Heights, IL) for 18 hours, harvested, and cell proliferation was evaluated by liquid scintillation counting.⁸

**Cytokine responses post-infection with either L. braziliensis or L. donovani.** At 14 (L. braziliensis-infected mice) or 80 (L. donovani-infected mice) days post-infection, two mice from the control and vaccinated groups were killed to identify the cytokines that were produced in response to infection. In L. braziliensis experiments, the popliteal and inguinal lymph nodes (that drained the footpad in mice infected with L. braziliensis) were harvested and crushed through a 20-μm pore size sieve, to obtain mononuclear cells. In L. donovani experiments, the spleens were used and splenic mononuclear cells were isolated as described above.

**Statistical analyses.** Statistical analyses were performed using Sigma Stat software (SPSS, Chicago, IL). Data for lesion progression were analyzed using analysis of variance for group comparisons and t-tests for other analyses. A P value < 0.05 was considered significant.

All experiments were performed at least three times. At least two mice were used for all time points in the proliferation (triplicate assay wells), cytokine (triplicate assay wells), limiting dilution (12 replicate assay wells) and, spleen weight analyses. At least four mice were tested for lesion development throughout the entire time course of infection with L. braziliensis.

**RESULTS**

**Lack of protection of BALB/c mice by vaccination with LmSEAg against a subsequent challenge with L. braziliensis.** We recently showed that LmSEAg administered in the absence of adjuvants are highly immunogenic molecules that protect BALB/c mice against challenge infection with L. major. In the work presented here, we sought to determine whether vaccination with LmSEAg could cross-protect BALB/c mice against L. braziliensis or L. donovani. In experiments in which mice were challenged with L. braziliensis, the mice were injected with 1 × 10⁶ stationary phase promastigotes subcutaneously in the left hind footpad. As shown in Figure 1, lesions tended to develop more slowly in the vaccinated mice, but overall there was no significant difference between lesion size in vaccinated mice compared with control mice (P > 0.05). This finding was corroborated by lesion parasite burden analyses, which at day 28 post-infection showed a 13-fold increase in mice that had received LmSEAg (Table 1).

**Reduction of splenic parasite burden by vaccination with LmSEAg after challenge with L. donovani.** Leishmania donovani persists in the spleen of BALB/c mice, with the concomitant development of considerable organ-specific pathology similar to that seen in human kala-azar. It was therefore important to evaluate the impact of vaccination in this organ. Immunized mice together with controls were challenged with 10⁶ stationary phase L. donovani promastigotes given intravenously through the tail vein. At days 7, 30, 60, and 80 after infection, two mice were killed and the increase in spleen weight was determined. In addition, parasite burdens in the spleens were determined. Figure 2 shows that although spleen weights in control mice increased over time, spleen weights in mice immunized with LmSEAg remained relatively constant. The differences in spleen weights between the control groups and LmSEAg-immunized mice were statistically significant (P < 0.05).

![FIGURE 1. Lack of stimulation of protective immunity in BALB/c mice after vaccination with Leishmania major promastigote exogenous antigens (LmSEAg) against a subsequent challenge with L. braziliensis.](image-URL)

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of infection</th>
<th>No. of L. braziliensis per footpad (95% confidence limits)</th>
<th>Fold change (+ or −) in parasite burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>70 (10–130)</td>
<td>NA</td>
</tr>
<tr>
<td>Control</td>
<td>28</td>
<td>310 (80–530)</td>
<td>NA</td>
</tr>
<tr>
<td>LmSEAg</td>
<td>14</td>
<td>70 (40–130)</td>
<td>No change</td>
</tr>
<tr>
<td>LmSEAg</td>
<td>28</td>
<td>4,010 (1,210–13,600)</td>
<td>(+) 13</td>
</tr>
</tbody>
</table>

*NA = not available; LmSEAg = L. major soluble promastigote exogenous antigens.
†Immunized as described in Materials and Methods.
‡On the indicated days post-infection, duplicate mice were killed and the parasite burdens in their footpads were analyzed as described in Materials and Methods.
Figure 2. Protection against spleen enlargement in BALB/c mice after vaccination with Leishmania major promastigote exogenous antigens (LmSEAs) against challenge with L. donovani. Mice were doubly immunized with LmSEAs alone. Thirteen days later, the mice were challenged with 10⁷ L. donovani stationary phase promastigotes intravenously through the lateral tail vein. At 7, 30, 60, and 80 days after infection, mice were killed and their spleen weights were determined. Spleen weights were significantly less in vaccinated than in control mice (P < 0.05). S.E. = standard error.

As shown in Table 2, reductions in spleen weights in LmSEAs-immunized mice were also accompanied by very low parasite burdens, as much as a 4,913-fold reduction in parasite burden in LmSEAs-immunized mice compared with controls (P < 0.001).

Proliferation and cytokines before infection. In an effort to delineate the mechanism(s) underlying the protection seen after challenge with L. donovani (but not L. braziliensis), we first determined what effect vaccination alone (in the absence of challenge with either L. donovani or L. braziliensis) had on the potential of the immune system to response to challenge with either LmSEAs, L. donovani, or L. braziliensis (Figures 3 and 4). Our results show that vaccinating induces cells that will proliferate in response to either parasite (but only modestly), and significantly to stimulation with LmSEAs (Figure 3).

Our previous experiments have shown that immunizing with LmSEAs induced a mixed Th1/Th2-type cytokine response in BALB/c mice. Once again, a mixture of IFN-γ and IL-4 were elicited by vaccination (Figure 4). Therefore, vaccination did not favor either a type 1 or type 2 response to either parasite. Thus, the reason(s) for this dichotomy is likely the result of events that occurred after infection with either parasite.

Production of elevated levels of type 2 cytokines in LmSEAs-immunized mice after infection with L. braziliensis. Fourteen days after immunized mice were challenged with L. braziliensis, mononuclear cells from the poplitic and inguinal lymph nodes (that drained the infected footpad) were harvested from mice and restimulated in vitro with either LmSEAs or with L. braziliensis promastigotes. As shown in Figure 5, the immunized mice produced approximately the same amount of IFN-γ as the controls, but produced greatly enhanced amounts of the type 2 cytokines IL-4 and IL-10 (P < 0.05).

Production of elevated levels of type 1 cytokines in LmSEAs-immunized mice after infection with L. donovani. We also investigated the nature of the immune response induced in mice immunized with LmSEAs, followed by infection with L. donovani. To achieve this, mononuclear cells were isolated from mouse spleens 80 days after challenge with L. donovani (the time when there were maximal differences in spleen weight and parasite burden (Figure 2 and Table 2). These cells were restimulated with either LmSEAs or L. donovani. Results showed that immunized mice produced elevated levels of IFN-γ (P < 0.05; Figure 6) and greatly reduced levels of IL-4 and IL-10 (P < 0.05; Figure 6). Therefore, in contrast to the experiments with L. braziliensis, immunized mice challenged with L. donovani produced a type 1 response to infection.

**DISCUSSION**

Resolution of an infection with a particular species of *Leishmania* usually confers complete resistance to re-
challenge with the same parasite. However, infection with a given species of *Leishmania* can also confer cross-protection against different species of *Leishmania*.\(^{16–28}\)

We recently showed that *Lm* SEAgs administered in the absence of adjuvants are highly immunogenic molecules that protect BALB/c mice against challenge infection with *L. major*.\(^8\) In this study, we sought to determine whether *Lm* SEAgs could cross-protect vaccinated BALB/c mice against infection with *L. braziliensis* or *L. donovani*. The BALB/c mouse is a widely used model for the study of the protective immune responses against these two parasites. In both infections, BALB/c mice develop type 1-based immune responses that leads to cure.\(^{28–31}\)

We found that injecting mice with *Lm* SEAgs elicited protection against a subsequent challenge with *L. donovani* (Figure 2 and Table 2), but not against a subsequent challenge with *L. braziliensis* (Figure 1 and Table 1). These observations were not due to an effect of vaccinating with *Lm* SEAgs that preferentially predisposed the vaccinated mouse to susceptibility to *L. braziliensis*, but resistance to *L. donovani* (Figures 3 and 4). Rather, vaccinating induced cells that proliferated in response to stimulation with either parasite (but only modestly), and significantly to stimulation with *Lm* SEAgs. With respect to cytokines produced, a mixture of IFN-γ and IL-4 were elicited in response to stimulation with either parasite after vaccination.

![Figure 4](image4.png)

**Figure 4.** Cytokine production in cells from BALB/c mice immunized with *Leishmania major* promastigote exogenous antigens (*Lm* SEAgs) in response to stimulation with *L. donovani* (*Ld*) and *L. braziliensis* (*Lb*). Mice were immunized with *Lm* SEAgs as described in the legend of Figure 3. Thirteen days later, spleen cells from duplicate immunized or control (injected with vehicle used to inject *Lm* SEAgs) mice were collected and pooled for cytokine assays. Mononuclear cell suspensions adjusted to a concentration of \(2 \times 10^6\) mL were prepared. These cells were stimulated *in vitro* with either \(2 \times 10^5\) *L. donovani/mL*, \(2 \times 10^5\) *L. braziliensis/mL*, or \(50 \mu g/mL\) of *Lm* SEAgs/mL. After two days, the culture supernatants were harvested and assayed for the presence of interleulin-4 (IL-4), IL-10, and IFN-gamma. S.E. = standard error.

![Figure 5](image5.png)

**Figure 5.** Cytokines produced by BALB/c mice vaccinated with *Leishmania major* promastigote exogenous antigens (*Lm* SEAgs) and challenged with *L. braziliensis*. Mice were immunized and challenged with *L. braziliensis*. Thirteen days after immunized mice were challenged, popliteal and inguinal lymph nodes that drained the infected footpad were harvested and restimulated *in vitro* with either *Lm* SEAgs (50 \(\mu g/mL\)) or *L. braziliensis* (\(2 \times 10^5/mL\)) promastigotes. Forty-eight hours later, supernatants of cultures were harvested and tested by enzyme-linked immunosorbent assay for IFN-gamma, interleukin-4 (IL-4), or IL-10. S.E. = standard error.

Therefore, these observations are likely the result of *Lm* SEAgs-treated mice not mounting a type 1 response when challenged with *L. braziliensis* (Figure 5), although they did mount this response when challenged with *L. donovani* (Figure 6). Why this occurred is a matter of debate. However, certain possibilities seem plausible, but they are thus far unproven. First, there could be something inherently different about *L. braziliensis* versus *L. donovani* and their interactions with the immune system. Alternatively, each of these parasites may differ in their ability to induce effector versus regulatory T cells. Further work is required to determine which, if either, of these possibilities might be correct.

It should be mentioned that *Lm* SEAgs-treated mice when challenged with *L. braziliensis* developed cutaneous lesions...
similar to those seen on control mice, but they also exhibited a 13-fold increase in the number of parasites present in their cutaneous lesions on day 28 of infection (Table 1). This may appear significant, but is probably not the case. As documented by Lima and others, when lesion sizes are different, differences in parasite burdens are frequently 100-fold to several 1,000-fold. Moreover, in Table 1 (experiments with L. braziliensis in which no protection was seen), the 95% confidence limits either overlap or are very close to the number of parasites per footpad. This is in contrast to the results in Table 2 (experiments with L. donovani in which substantial protection was observed) where the confidence limits are vastly different from each other.

We have consistently found LmSEAgS to be highly immunogenic. The precise reasons for their immunogenicity are not known. Moreover, soluble antigens are frequently poorly immunogenic and can induce tolerance. We have suggested that the immunogenicity of LmSEAgS might be related to the lipophosphoglycan present or contaminating lipopolysaccharide. Like lipopolysaccharide, lipophosphoglycan can activate the innate immune system through interaction with toll-like receptors and this in turn strongly activates the specific immune system.

Taken as a whole, these highly-immunogenic and protective qualities of LmSEAgS suggest that they might be a promising vaccine target for Old World cutaneous and visceral leishmaniasis. The group of Reed and others is currently developing a polyprotein vaccine that incorporates secreted antigens of Leishmania. Their approach is different from the approach described here; however, yet both approaches are proving to be quite successful.

Received July 18, 2006. Accepted for publication November 27, 2006.

Acknowledgments: We thank Sheryl Carter and Jeanette V. Bishop for technical assistance, and Lisa P. Hochberg, Jeffrey R. Ryan, and Samuel K. Martin for help in many facets of this project.

Financial support: This study was supported by The Medical Research and Materiel Command, Military Infectious Diseases Research Program, Contract number DAMD17-01-P-0237 and National Institutes of Health AI 027511, AI029955, and AI 065784.

Authors’ addresses: Willy K. Tonui, Centre for Biotechnology Research and Development, Kenya Medical Research Institute, PO Box 54840, Nairobi 00200, Kenya; Telephone: 254-020-2722541(4), E-mail: wtonui@kemri.org. Richard G. Titus, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1619, Telephone: 970-491-4964, Fax: 970-491 0603, E-mail: rtitus@colostate.edu.

REFERENCES

Figure 6. Cytokines produced by BALB/c mice vaccinated with Leishmania major promastigote exogenous antigens (LmSEAgS) and challenged with L. donovani. Mice were immunized with LmSEAgS alone. Eighty days after immunization, mice were challenged with L. donovani, and mononuclear cells from their spleens were restimulated in vitro with LmSEAgS (50 μg/mL) or with L. donovani (2 × 10^7/mL) promastigotes. Forty-eight hours later, supernatants of cultures were harvested and tested by enzyme-linked immunosorbent assay for IFN-gamma, interleukin-4 (IL-4), or IL-10. S.E. = standard error.


