Protection of Susceptible BALB/c Mice from Challenge with *Leishmania major* by Nucleoside Hydrolase, a Soluble Exo-antigen of *Leishmania*

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**Abstract.** *Leishmania major* culture-derived, soluble, exogenous antigens have been shown to be a source of vaccine targets for the parasite. We have previously reported that *L. major* culture-derived, soluble, exogenous antigens can immunize BALB/c mice against challenge with *L. major*. However, the molecule(s) involved in this protection was not known. We describe the potential of one component of soluble exogenous antigens (recombinant nucleoside hydrolase) to vaccinate mice against challenge with *L. major*. We found that recombinant nucleoside hydrolase vaccinated BALB/c mice against a subsequent challenge with *L. major*. Protection was manifested by a significant decrease in lesion size (as much as a 30-fold reduction) and parasite burden (as much as a 71-fold reduction). Protection was achieved whether recombinant nucleoside hydrolase was administered to mice in the presence or absence of adjuvant (interleukin-12). Finally, protection was accompanied by an increase in interferon-γ production but a decrease in interleukin-10 production by vaccinated animals in response to challenge with *L. major*.

INTRODUCTION

It is now widely believed that the outcome of infection with *Leishmania major* in mice is largely determined by whether the parasite elicits a Th1 or Th2 response. In most mouse strains (e.g., C57BL/6, C3H, CBA), a Th1 response prevails. The production of high levels of interferon-γ (IFN-γ) that accompanies this response activates *L. major*-infected macrophages to kill the parasite, which enables the mice to recover from infection. In contrast, BALB/c mice mount a Th2 response, which is ineffective because only low levels of IFN-γ are produced. Moreover, high levels of interleukin-4 (IL-4) and IL-10 produced by BALB/c mice infected with *L. major* may block the ability of IFN-γ to activate parasitized macrophages. As a result, BALB/c mice die of infection with *L. major*.

Interleukin-12 is a cytokine that plays a protective role in *L. major* infection. Infusing IL-12 into susceptible BALB/c mice prior to infection with *L. major* allows the mice to develop a protective Th1 response to the parasite and to cure their infection. Interleukin-10 is a cytokine that plays an exacerbative role in *L. major* infection, and it is thought to prolong infection with *L. major* in mice and to be involved in the lifelong immunity to reinfection with *L. major* that occurs after resolution of the initial infection with the parasite.

We have recently observed that soluble *L. major* exo-antigens (*LmnSEAs*) in the absence of any adjuvant are immunogenic and induce protection (both with respect to lesion size and parasite burden within lesions) in susceptible adult BALB/c mice to challenge with *L. major*. We investigated whether a single component of SEAs (a protein present in *L. donovani* SEAs that was produced in recombinant form) could vaccinate BALB/c mice against challenge with *L. major*. We found that this protein (*L. donovani* recombinant nucleoside hydrolase [rNuH]) induced marked protection in susceptible BALB/c mice and that the protective potential of NuH was not enhanced by co-injection with IL-12. Finally, protection after vaccination with rNuH correlated with increased production of IFN-γ and decreased production of IL-10. These results suggest that NuH should be considered as a component of subunit vaccines against leishmaniasis.

MATERIALS AND METHODS

**BALB/c mice.** Young, adult, female BALB/c mice 4–6 weeks of age were bred at Laboratory Animal Resources (Colorado State University, Fort Collins, CO) and if not available they were obtained from the National Cancer Institute (Bethesda, MD). In most experiments, groups of 10 mice each were used. This allowed for four mice to be followed for lesion size throughout the experiment, and three subgroups of duplicate mice each that could be used for assays to determine parasite burden and the nature of the immune response.

**Leishmania major parasites.** Metacyclic promastigotes of *L. major* (strain MRHO/SV/59/LV39) were used. Parasites were maintained as previously described. Briefly, parasites were obtained from infected footpads of C3H mice and cultured in Novy-MacNeal-Nicolle medium for five days. After five days, metacyclic promastigotes were isolated from stationary phase cultures using peanut agglutinin.

**Preparation of rNuH and lipopolysaccharide.** Recombinant NuH was produced according to published techniques. Briefly, the open reading frame of the *L. donovani* NuH gene was cloned into the bacterial expression vector pET 22b(+), and the recombinant enzyme was expressed in *Escherichia coli* strain BL21. Six histidine residues were included at the carboxyl terminus to allow purification of the recombinant NuH using nickel columns. *Escherichia coli* lipopolysaccharide (LPS, 055 B5W) was obtained from Difco (Detroit, MI). The degree of contamination of LPS in the NuH preparation was determined by a *Limulus* amebocyte lysate assay for endotoxin according to manufacturer’s directions (Associates of Cape Cod, East Falmouth, MA).

Establishing the appropriate dose for vaccination with rNuH and challenge with *L. major*. BALB/c mice were immunized with various doses of rNuH alone or with rNuH in conjunction with recombinant murine IL-12 (rmlIL-12) (Genetics Institute, Cambridge, MA) subcutaneously in the

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rump. Thirteen days later the immunizations were repeated. Controls either received vehicle alone or rmIL-12 alone. A dose of 1 µg of rmIL-12 per mouse was used because this concentration of rmIL-12 was shown by Afonso and others\textsuperscript{13} to act as an adjuvant for a whole lysate of \textit{L. major}. Seven days after the second immunization, the mice were challenged with 10\textsuperscript{6} metacyclic \textit{L. major} in one hind footpad. Lesion development was followed by measuring the thickness of the infected footpad compared with the thickness of the same footpad prior to infection. Parasite numbers were determined in infected footpads using a published limiting dilution assay for determining parasite burdens in infected mouse footpads.\textsuperscript{14}

Stimulation of cells from immunized BALB/c mice. Lymphatic tissue (the spleen or the popliteal and inguinal lymph nodes that drained the footpad in mice infected with \textit{L. major}) was harvested from duplicate mice of each experimental group just prior to infection (day 0) or at 20 days post-infection. When spleen cells were used, mononuclear cells were purified using Ficoll gradients (Sigma, St. Louis, MO).

Mononuclear cells were adjusted to a concentration of 2 × 10\textsuperscript{5}/mL in Dulbecco’s modified Eagle medium\textsuperscript{15} containing 0.5% normal mouse serum (Harlan Bioproducts, Indianapolis, IN) and 5 × 10\textsuperscript{-5} M 2-mercaptoethanol (Sigma). Cells were stimulated \textit{in vitro} with 50–100 µg of rNuH/mL, \textit{L. major} (2 × 10\textsuperscript{5} metacyclic \textit{L. major}/mL), or medium alone. Supernatants were collected after 48 hours of culture and tested by enzyme-linked immunosorbent assay for IL-4, IL-10, and IFN-\textgamma by commercial anti-cytokine antibody pairs (Becton Dickinson/Pharmingen, San Jose, CA) and protocols provided by the manufacturer.\textsuperscript{16}

In some cases, after five days the cultures were pulsed with 1 µCi of \textsuperscript{3}H-thymidine (5 Ci/mmol; Amersham, Arlington Heights, IL) for 18 hours, harvested, and cell proliferation was evaluated by liquid scintillation counting (Tomtec cell harvester/Wallac liquid scintillation counter; Long Island Scientific, Port Jefferson, NY).

Statistical analyses. Statistical analyses were performed using Sigma Stat (SPSS, Chicago, IL). Data for lesion progression were analyzed using analysis of variance for group comparisons and \textit{t}-tests for cytokine production and cell proliferation. Overall, results were considered significant when the \textit{P} was < 0.05. All experiments were performed at least twice.

RESULTS

Protection of susceptible BALB/c mice from a subsequent challenge with \textit{L. major} by immunization with rNuH alone. BALB/c mice were immunized by injecting various doses of soluble rNuH in the absence of adjuvant into susceptible BALB/c mice. The mice were immunized subcutaneously in the rump, and 13 days later the immunizations were repeated. Controls received vehicle alone (phosphate-buffered saline). Seven days after the second immunization, the mice were challenged with 10\textsuperscript{5} metacyclic \textit{L. major} in one hind footpad. Lesion development was followed by measuring the increase in the thickness of the challenged footpad compared with the same footpad prior to infection. As is shown in Figure 1, all doses of rNuH imparted marked protection against a subsequent challenge with \textit{L. major}. Therefore, in subsequent experiments an intermediate dose of rNuH (5 or 10 µg) was used. In some experiments, mice were vaccinated with rNuH more than two times, but the degree of protection was no greater than that shown in Figure 1. Analysis of variance showed that lesion sizes of all vaccinated groups in Figure 1 were significantly smaller (\textit{P} < 0.05) compared with lesions that developed in control mice.

Effect of IL-12 as an adjuvant on the vaccinating potential of rNuH. We next determined whether including an adjuvant such as IL-12 could enhance the vaccine potential of rNuH. A dose of 1 µg of rmIL-12 per mouse was used because this concentration of rmIL-12 has been previously determined by Afonso and others\textsuperscript{13} to act as an adjuvant for a whole lysate of \textit{L. major}. Otherwise, the vaccine regimen was as described in Figure 1 and the Materials and Methods. As shown in Figure 2, the vaccine potential of rNuH was not enhanced by IL-12. Indeed, 5 µg of rNuH was just as protective as 5 µg of rNuH plus IL-12 (Figure 2).

Parasite burden in susceptible BALB/c mice immunized with rNuH and challenged with \textit{L. major}. Tracking lesion size (as a measure of the pathologic response to infection) is one method of monitoring the development of infection with \textit{L. major}. Arguably, a better method is tracking the parasite burden in control or vaccinated mice challenged with \textit{L. major}. As shown in Table 1, the parasite burden was markedly reduced in mice pretreated with rNuH (with or without IL-12) and subsequently challenged with \textit{L. major} (a 12–71-fold reduction in parasite numbers, Table 1). The greatest reduction in parasite burden (71-fold) was seen in mice vaccinated with 5 µg of rNuH alone in the absence of adjuvant (Table 1).

Characterizing the nature of the immune response in mice vaccinated with rNuH prior to challenge with \textit{L. major}. As shown in Table 2, there were lymphoid cells in rNuH-
vaccinated mice that had been primed to respond to stimulation with rNuH. First, lymphoid cells drawn from rNuH-immunized mice proliferated in response to stimulation with rNuH (Table 2). Moreover, these lymphoid cells produced substantial amounts of IL-10 in response to stimulation with rNuH (Table 2).

Characterizing the nature of the immune response in mice vaccinated with rNuH after challenge with *L. major*. We then determined the nature of the immune response in mice that had been vaccinated with rNuH and then challenged with *L. major* for 20 days (Table 3). Twenty days of infection was chosen for these assays because by 20 days clear, significant, and consistent differences had developed in the size of lesions of control mice compared with vaccinated mice (Figures 1–3).

Therefore, the cytokines produced by these different groups of mice should be distinguishable from each other and should suggest a mechanism of protection in rNuH-treated mice. Lymphoid cells drawn from control (non-vaccinated) mice produced low levels of IFN-γ whether they were restimulated with rNuH or *L. major* promastigotes *in vitro* (Table 3). In contrast, vaccinated mice produced substantially more IFN-γ when restimulated *in vitro* with either rNuH (> 39-fold) or *L. major* (3-fold, Table 3).

The observations made with IL-10 were the opposite of those seen with IFN-γ (Table 3). Lymphoid cells from control mice not vaccinated with rNuH produced substantial amounts of IL-10 in response to stimulation with either rNuH or *L. major* *in vitro* (Table 3). However, when mice were vaccinated with rNuH and lymphoid cells from the mice were challenged with rNuH or *L. major* *in vitro*, the cells produced undetectable amounts of IL-10 (Table 3).

We also measured IL-4 production. Only low and inconsistent levels of IL-4 were detected; thus, results with this cytokine are not shown. This lack of IL-4 production is not surprising because the explanation for the susceptibility of BALB/c mice to infection with *L. major* is complex. For example, in certain cases IL-4-deficient BALB/c mice (e.g.,17) are still susceptible to infection with the parasite. This observation may in part be explained by the fact that IL-4-deficient BALB/c mice are more or less susceptible to infection with *L. major* depending upon the substrain of parasite used to infect the mice because some strains of *L. major* induce infection in IL-4-deficient BALB/c mice while others do not. Thus, the susceptibility of BALB/c mice to infection with *L. major* may be dependent upon the ability of the parasite to induce not only the production of IL-4, but also other cytokines such as IL-10,18,19 which also promotes susceptibility to infection with the parasite.

**Effect of LPS that contaminates the rNuH preparation on protection of BALB/c mice from challenge with *L. major***. Because rNuH was a recombinant protein produced in *E. coli*,15 LPS that contaminated this preparation could have been involved in the protection that was observed in mice vaccinated with rNuH. Therefore, we determined the amount of LPS present in the preparation and its ability to substitute for rNuH in protecting mice from challenge with *L. major*.

![Figure 2. Lack of enhancement by interleukin-12 of the protective effect of recombinant nucleoside hydrolase (rNuH) in susceptible BALB/c mice.](image)

**Table 1. Parasite burdens in footpad lesions**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day post-infection</th>
<th>Parasite burden × 10⁶ (95% confidence limits)</th>
<th>Fold decrease in parasite burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>37.3 (11.3–63.4)</td>
<td>NA</td>
</tr>
<tr>
<td>5 µg rNuH</td>
<td>28</td>
<td>1.1 (0.3–1.9)</td>
<td>33.0</td>
</tr>
<tr>
<td>1 µg rIL-12</td>
<td>28</td>
<td>6.5 (2.0–10.9)</td>
<td>5.8</td>
</tr>
<tr>
<td>1 µg rIL-12 + 5 µg rNuH</td>
<td>28</td>
<td>3.2 (1.2–5.3)</td>
<td>11.5</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>85.3 (50.6–120.6)</td>
<td>NA</td>
</tr>
<tr>
<td>5 µg rNuH</td>
<td>42</td>
<td>1.2 (0.7–1.7)</td>
<td>70.5</td>
</tr>
<tr>
<td>1 µg rIL-12</td>
<td>42</td>
<td>7.7 (4.9–10.6)</td>
<td>11.0</td>
</tr>
<tr>
<td>1 µg rIL-12 + 5 µg rNuH</td>
<td>42</td>
<td>3.6 (1.0–6.2)</td>
<td>23.8</td>
</tr>
<tr>
<td>Control</td>
<td>28</td>
<td>46.3 (26.3–66.3)</td>
<td>NA</td>
</tr>
<tr>
<td>5 µg rNuH</td>
<td>28</td>
<td>1.8 (1.5–2.1)</td>
<td>25.7</td>
</tr>
<tr>
<td>17 pg LPS</td>
<td>28</td>
<td>52.6 (34.4–70.8)</td>
<td>None</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>77.7 (64.8–90.6)</td>
<td>NA</td>
</tr>
<tr>
<td>5 µg rNuH</td>
<td>42</td>
<td>0.9 (0.6–1.2)</td>
<td>86.3</td>
</tr>
<tr>
<td>17 pg LPS</td>
<td>42</td>
<td>69.9 (45.6–94.2)</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Controls were injected with the diluent used to inject the rNuH, IL-12, or LPS.

rNuH = recombinant nucleoside hydrolase; IL-12 = interleukin-12; NA = not applicable; rIL-12 = recombinant murine IL-12; LPS = lipopolysaccharide.
of LPS that was present in our rNuH preparation by the Limulus amebocyte lysate assay for endotoxin and found it to be 17 pg/5 μg of rNuH. We then treated mice with either 5 μg or 10 μg of rNuH for 17 pg of LPS according to the protocol outlined in Figure 1 and the Material and Methods. As shown in Figure 3, purposely injecting 17 pg of LPS had no protective effect, whereas injecting 5 μg of rNuH was strongly protective in treated mice. Corroborating these lesion size results, we also found that LPS did not affect the number of parasites present in the lesions (Table 1). The degree of parasite reduction in lesions was almost identical whether the rNuH-vaccinated group was compared with control mice or LPS-treated mice: 25.7–29.2-fold at day 28 of infection, and 77.7–86.3-fold at day 42 of infection. Therefore, the LPS contaminating the rNuH preparation was not responsible for the protection observed in mice vaccinated with rNuH.

**DISCUSSION**

We have previously reported that vaccinating with SEAgs can protect susceptible BALB/c mice against challenge with *L. major*. However, the molecules(s) responsible for this protection was unknown. In this report (Figure 1 and Table 1), we show that a single protein, rNuH, can substitute for SEAgs and protect susceptible BALB/c mice from challenge with *L. major*. We selected NuH as the immunogen that would most likely substitute for SEAgs because our work has shown that NuH is a very immunogenic component of SEAgs.

The *L. donovani* NuH used in this study was isolated by immunoscreening an *L. donovani* DNA library using polyclonal antibodies against *L. donovani* SEAgs. In dogs naturally infected with leishmaniasis, there are high antibody titers against NuH protein. It is important to also note that the fucose-mannose ligand is an antigenic complex from promastigotes of *L. donovani* that is used for diagnosis, prognosis, and blood-bank control of human leishmaniasis, and NuH has been identified as a major component in this complex. In addition to this study, NuH has been found to be highly antigenic in different animals (mice, rabbits, and dogs). Therefore, it would be interesting to determine whether this protein is also antigenic in humans, and if it is, this would suggest that it has diagnostic and vaccine potential for humans.

Nucleoside hydrolase NuH is a highly conserved protein in all parasite species that have been examined. This is likely because it is an enzyme that parasites use to salvage nucleotides from their environment and thus is required for the synthesis of parasite DNA. In the work presented here, *L. donovani* NuH protected against challenge with *L. major*. Other investigators have reported that *L. donovani* NuH can also protect mice against challenge with *L. mexicana* or *L. donovani*. This suggests that this enzyme could be used as a vaccine candidate for many if not all *Leishmania* species. It is interesting that NuH is also a target for designing antipROTOZOAN parasite drugs.

Vaccinating with rNuH was able to protect susceptible BALB/c mice from infection with *L. major*, in many cases at six weeks of infection these mice had no detectable lesion pathology (Figures 1 and 2). It was not ethical to follow the infections beyond six weeks because at this point the lesions of BALB/c mice contain massive numbers of parasites (> 100 x 10^6 parasites/footpad), and ultimately the animals will die of the infection unless they are treated with drugs and/or cytokines. It is also interesting that including IL-12 in the
inoculum with rNuH was no more (and in some cases less effective, Table 1) effective than injecting rNuH alone.

Historically, injecting soluble antigen can lead to the induction of tolerance, i.e., the lack of an immune response to antigen. However, although rNuH was administered in soluble form, it was highly protective (Figures 1 and 2 and Table 3).

In general, after infection with L. major, control mice gravitated towards an enhanced type 2 (IL-10) immune response, with no increase in a type 1 response (IFN-γ). In contrast, immunizing with rNuH showed the opposite: up-regulation of a type 1 response, but no effect on a type 2 response (Tables 2 and 3). This may be the reason immunization with NuH protected against challenge with L. major. Induction of a type 1 response, as opposed to a type 2 response, has consistently been associated with protection against infection with L. major.1–5

It is important that LPS was not responsible for the protection observed with NuH (Figure 3 and Table 1). In our previous publication that showed that immunization with SEAgs alone could protect susceptible BALB/c mice from infection with L. major, we speculated that the lipophosphoglycan present in the SEAgs preparation might be responsible for the protection observed in mice vaccinated with SEAgs.6

Our reason for this speculation is that, like LPS, lipophosphoglycan can activate the innate immune system through interaction with toll-like receptors,7–10 and activation of the innate immune system is also a strong activator of the specific immune system. However, because LPS could not substitute for NuH, and the rNuH preparation would not have contained lipophosphoglycan, it appears that SEAgs/rNuH might be inherently immunogenic by a mechanism that has not yet been defined. It is important to note that to date no adjuvant effect of NuH has been reported. Alternatively, although LPS could not provide protection, this does not rule out the possibility that NuH synergizes with LPS to bring about the protection seen.

The results presented in this study suggest that SEAgs, in particular NuH, should be considered as a component of subunit vaccines for controlling leishmaniasis. In addition to this report, Aguilar-Be and others22 and Gamboa-Leon and others23 have shown that NuH (administered as a DNA vaccine and/or with adjuvant) can protect BALB/c mice against infection with either L. donovani or L. mexicana. This report shows that NuH will also protect BALB/c mice against infection with either L. donovani or L. mexicana. This report shows that NuH will also protect BALB/c mice against infection with either L. donovani or L. mexicana. This report shows that NuH will also protect BALB/c mice against infection with either L. donovani or L. mexicana.

In conclusion, NuH is an excellent target for vaccine development because it is conserved among Leishmania parasites, is essential for salvage of purines, is highly immunogenic in different animals, and is protective against infection with several species of Leishmania (if not all) in mice. And perhaps most importantly, at the protein level, there are no structures in humans that are homologous to L. donovani NuH. A search at the National Center for Biotechnology Information website showed that there are no homologs of L. donovani NuH in any mammals, including humans. Homologs of L. donovani NuH are found only in bacteria and protozoa, not in metazoa. Therefore, NuH is an ideal choice of antigens to vaccinate against leishmaniasis because it should be immunogenic but it should not induce autoimmunity in humans.

Received June 26, 2007. Accepted for publication August 22, 2007

Acknowledgments: We thank Jeremy Jones, Leanna Nobisch, and Jeanette Bishop for expert technical assistance.

Financial support: This study was supported in part by the Medical Research and Materiel Command, Military Infectious Diseases Research Program, contract number DAMD17-01-P-0237 and National Institutes of Health grants AI 27511, AI 29955, and AI 065784. Mohammad A. Al-Wabel was supported by a scholarship from Qassim University (Buraydah, Al-Qaseem, Saudi Arabia).

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