Antithymocyte Serum Suppression of Immunity in Mice Immunized to *Leishmania donovani*

G. L. RUNEY, Ph.D.,† J. C. JARECKI-BLACK, Ph.D.,‡§ M. W. RUNEY, Ph.D.,¶ and A. B. GLASSMAN, M.D.¶

†Department of Biology, The Citadel, Charleston, SC 29425
and
‡Department of Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425
and
¶Department of Biology, College of Charleston, Charleston, SC 29425

ABSTRACT

Mice immunized with a glucan-killed parasite vaccine exhibited enhanced resistance to *Leishmania donovani* infection as evidenced by decreased hepatic parasite burdens when compared to unvaccinated controls. This resistance was not seen in mice immunized with killed parasites alone. Glucan vaccination resulted in increased resistance at day 6, but this effect was no longer present by day 20 of the experiment. Treatment of vaccinated and control mice with antithymocyte sera abrogated protection against infection, whether such resistance was vaccine induced or the result of acquired immunity.

Introduction

Visceral leishmaniasis, caused by the protozoan parasite *Leishmania donovani*, is a significant cause of mortality and morbidity worldwide. Current therapy relies upon chemotherapeutic intervention with pentavalent antimonials, but such drugs are relatively toxic and treatment failure of 10 to 25 percent has also been reported. Recent research has resulted in the development of potential new drug regimens and approaches to treatment, but vaccine-induced resistance would provide a powerful alternative in the control of visceral leishmaniasis. Our laboratory has previously reported protection in mice induced with a subcutaneous immunization protocol combining formalin killed parasites.
administered with glucan.\(^6\) It has also been shown by us that the resistance elicited with this vaccine can be transferred to naive, syngeneic mice with \(5 \times 10^8\) spleen cells from immunized mice.\(^8\) In an attempt to determine if T-lymphocytes were responsible for the increased resistance observed in vaccinated mice and in recipients of adoptively transferred spleen cells, antithymocyte serum (ATS) were used specifically to suppress cell mediated immunity.

**Materials and Methods**

**Experimental Model and Parasite**

Female C57BL/6 mice, eight to 10 weeks of age, were obtained commercially\(^*\) and maintained in standard caging under the supervision of a veterinarian. A commercial mouse diet\(†\) was provided *ad libitum*.

*Leishmania donovani*, strain WR371, was maintained in our laboratory in axenic culture. Growth media consists of Media 199 supplemented 10 percent with lysed, defibrinated rabbit blood and 14.6 mg L-glutamine per litre. Cultures were initiated from minced mouse spleen and passed at four-to-five day intervals. Promastigotes for immunization or challenge were harvested within 10 serial passages of culture initiation.

**Immunization Protocol**

Promastigotes for immunization or challenge were harvested during stationary phase growth by centrifugation (900 g, 15 min) as a previous study has shown that stationary-phase parasites are more immunogenic,\(^9\) as well as more infective than those harvested from log phase cultures. The parasites were washed three times in Earles Balanced Salt Solution (EBSS), and killed in 0.1 percent formalin for 30 minutes at room temperature. Formalin killed parasites (P) were kept at 4°C overnight, washed three times, counted in a hemocytometer and resuspended in EBSS at a concentration of \(10^8\) per ml. Glucan was prepared as described previously.\(^4\) Mice were divided into groups (14 mice each) and immunized according to a standard protocol, consisting of three subcutaneous injections given at four-day intervals. Groups received either \(10^7\) formalin killed parasites combined with six mg glucan (GP), glucan alone (G), dead parasites alone (P), or were untreated (NT). Half of the mice in each group were treated with antithymocyte serum.\(‡\) Three subcutaneous injections, two ml each, were given on an alternate day basis for the first week and then continued twice weekly for the duration of the experiment. All mice were challenged intravenously 21 days post-immunization with \(10^7\) promastigotes per mouse.

**Monitoring of Infection Course**

Groups of mice (7 mice each) were sacrificed in chloroform vapor at day 6 and day 20 post-challenge. Body, spleen, and liver weights were recorded, and impression slides were made from liver and spleen. Slides were fixed with methanol, stained with giemsa, and hepatic parasite burdens determined according to Staube's method.\(^19\) The hepatic amastigote burden was used as the indicator of resistance since splenic parasites are rapidly cleared in this model. Results were analyzed using the Mann-Whitney U-test\(^10\) with a probability of \(p < 0.05\) considered significant. All data were expressed as mean \(\pm\) standard error of the mean.

---

\(^*\) Jackson Laboratory, \(^?\)

\(†\) Purina.

\(‡\) Calbiochem-Behring Corp., La Jolla, CA.
Results

Mice immunized with the glucan and killed parasite vaccine (GP) demonstrated significant reductions (p < 0.02) in hepatic parasite burdens as compared to untreated mice at both day 6 and day 20 post-challenge (table I). Mice receiving killed parasites alone (P) at either experimental date or vaccinated with glucan (G) alone at day 20 were not protected against infection with *L. donovani*.

Parasite burdens in mice vaccinated with glucan alone were lower than those in untreated mice (p < 0.05) at day 6. This is consistent with previous results in our laboratory which show that the administration of glucan, a non-specific immunomodulator, may result in a transitory increase in immune responsiveness in the early stages of infection. The protection induced by the GP vaccine, however, is consistent and is seen throughout the course of the experiment. By day 20 post-challenge, results indicate there is no longer any difference in parasite burdens between glucan treated mice and controls.

Administration of anti-thymocyte sera appears to abrogate the vaccine-induced resistance seen in GP mice. Parasite burdens are significantly increased in GP vaccinated animals receiving ATS (p < 0.001) at both day 6 and day 20 post-challenge. Comparison of GP vaccinated mice treated with ATS (GPats) to untreated controls also receiving ATS (NTats) shows that at day 6, NTats animals exhibited significantly fewer parasites (p < 0.001) than GPats mice. By day 20, however, parasite burdens did not differ in the two groups. Untreated controls exhibited no differences in parasite proliferation at day 6, whether or not the mice were treated with ATS. However, by day 20, NTats mice showed higher levels of infection than untreated mice not receiving ATS.

Discussion

Infection with *Leishmania donovani* is characterized by a wide range of host immunological responses. In man, leishmaniasis often leads to unrestricted parasite proliferation, with death being the eventual outcome. Chemotherapeutic regimens can interrupt this process leading to the acquisition of a strong immune response which prevents reinfection with the homologous species of parasite. Current evidence indicates that cell mediated immunity (CMI) is responsible for the acquired resistance to visceral leishmaniasis seen in spontaneous or drug cured individuals.

Although the C57/BL mouse is considered to be a valid model for the study of experimental leishmaniasis, infection with *L. donovani* is eventually cured, even in untreated mice. Such acquired resistance is genetically determined. In our hands, peak parasite levels are seen about 14 days post-challenge and then

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 6</th>
<th>Day 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP</td>
<td>27.43 ± 3.70</td>
<td>34.73 ± 9.23</td>
</tr>
<tr>
<td>GPats</td>
<td>101.73 ± 22.90</td>
<td>681.56 ± 156.28</td>
</tr>
<tr>
<td>G</td>
<td>33.94 ± 0.10</td>
<td>159.04 ± 13.31</td>
</tr>
<tr>
<td>Gats</td>
<td>65.42 ± 15.43</td>
<td>548.51 ± 40.08</td>
</tr>
<tr>
<td>P</td>
<td>44.78 ± 4.35</td>
<td>109.49 ± 23.41</td>
</tr>
<tr>
<td>Pats</td>
<td>52.65 ± 8.56</td>
<td>517.02 ± 64.06</td>
</tr>
<tr>
<td>NT</td>
<td>45.42 ± 5.40</td>
<td>150.21 ± 44.44</td>
</tr>
<tr>
<td>NTats</td>
<td>38.29 ± 9.94</td>
<td>420.76 ± 58.06</td>
</tr>
</tbody>
</table>

Mice received three subcutaneous immunizations at four day intervals combining glucan and killed parasites (GP); glucan alone (G); killed parasites alone (P) or were untreated (NT). Mice received 0.2 ml anti-thymocyte sera (ATS) on alternate days throughout the course of the experiment. N = 7 animals per group; data is given as the mean ± standard error of the mean.
slowly are reduced until parasites can no longer be detected in liver impression smears.

Our laboratory has previously reported that resistance against *L. donovani* infection in mice can be induced with a subcutaneous immunization protocol combining glucan and formalin killed promastigotes as evidenced by a decrease in parasite levels during peak infection. It has also been reported by us that the transfer of such vaccine induced resistance is possible by injecting naive, syngeneic recipients with $5 \times 10^8$ spleen cells harvested from immunized donors, thus indicating that CM1 may be important in murine leishmaniasis as has been previously suggested. In order to determine if such vaccine induced immunity is solely dependent upon T-lymphocytes, it was attempted in our laboratory specifically to suppress cell mediated immunity by injecting antithymocyte serum (ATS) after vaccination and throughout the course of the experiment. Immunosuppression with ATS has been shown to result in the growth or increase in the number of metastases in a variety of human and animal malignant tumors and in the promotion of successful organ transplantation.

Further results reveal that the administration of ATS significantly increases parasite burdens in mice previously vaccinated with the GP immunization protocol at both days 6 and 20 of the experiment. By day 20 post-challenge, this increase in amastigote proliferation is apparent in all groups treated with ATS. It is possible that ATS administration only affected the GP group because this group alone demonstrates increased resistance to infection, presumably through the stimulation of cell-mediated immunity. Groups receiving killed parasites alone or untreated mice do not show increased resistance to infection at this early date. By day 20, however, all mice are beginning to mount an immune response to *L. donovani*, whether as a result of vaccine induced resistance or acquired immunity. Such immunity may thus be affected by ATS and result in the increased parasite burdens seen in our results.

The use of ATS provides a specific probe for the suppression of cell-mediated immunity. Most of the evidence concerning infection by *leishmania* organisms indicates that cell mediated immunity, in general, and T-lymphocytes, in particular, are responsible for resistance to leishmaniasis. However, such a hypothesis does not rule out a role for B-lymphocytes. Alexander and Phillips showed that the transfer of both T and B-lymphocytes provided optimum protection against *L. mexicana* while B cells alone conferred no protection to infection. With *L. major*, however, the transfer of T-cells alone was as effective at conferring resistance as the transfer of B and T-lymphocytes.

This study provides evidence substantiating the role of T-lymphocytes in promoting immunity against visceral leishmaniasis, whether or not such immunity is acquired as a result of infection or is induced by a vaccination protocol.

Acknowledgments

The authors gratefully acknowledge the help and encouragement of the late Dr. Thomas W. Holbrook, without whom this project would never have reached completion, and the editorial assistance of Pam Eidson. The studies were supported by UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

References


