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VACCINATION OF Rhesus Monkeys AGAINST Plasmodium

KNOWLESI WITH LYOPHILIZED ANTIGEN.

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ABSTRACT

Twenty-two Rhesus monkeys were used to determine the efficacy of non-viable lyophilized \textit{Plasmodium knowlesi} antigen as an immunizing material. Fourteen monkeys were vaccinated twice with lyophilized or unlyophilized antigen, while eight animals served as unvaccinated controls. After challenge with the homologous \textit{P. knowlesi} strain all of the control monkeys succumbed to the infection, while 8 of 14 vaccinated monkeys survived. Those vaccinated animals that died did so significantly later than did the controls. The prepatent period was significantly shorter in control animals than in vaccinated animals.
INTRODUCTION

Vaccination of Rhesus monkeys against *Plasmodium knowlesi* by use of non-viable *P. knowlesi* blood stage antigen has been described.\(^1,\) \(^2\) Protection has also been reported after vaccination with intact blood stage plasmodial parasites.\(^3\)-\(^5\)

The need for a vaccine, suitable for shipment and long-term storage in tropical areas led to the present study examining the effect of lyophilization on the protectivity of non-viable antigen. In the only other report on the use of lyophilized malarial antigen, one monkey was sensitized with lyophilized schizonts of *P. knowlesi*, and found to be protected upon intradermal challenge.\(^5\)

MATERIALS AND METHODS

**Antigen Production.** Antigen was prepared from *P. knowlesi* (H strain) infected Rhesus monkey blood as described.\(^1\) Immediately after antigen collection, protein determinations were made by the Lowry Method.\(^6\) The antigen was either lyophilized, or quick frozen and stored in liquid \(\text{N}_2\). Lyophilized antigen was reconstituted in sterile distilled \(\text{H}_2\text{O}\) immediately prior to use.

**Animals.** Twenty-two female Rhesus monkeys (*Macaca mulatta*) ranging in weight from 3.9 to 5.4 kg were divided into 6 groups. One group served as an unvaccinated control, while a second group received adjuvant with phosphate buffered saline (PBS) given on the same schedule as the antigen injected group. Three groups received lyophilized antigen given in doses of either 250 \(\mu\)g, 500 \(\mu\)g, or 1500 \(\mu\)g per animal per injection. An additional group received 1000 \(\mu\)g of non-lyophilized antigen per animal per injection.
Adjuvant. All antigen was emulsified in adjuvant immediately prior to use. For the first injection the antigen was emulsified in an equal volume of Freund's Complete Adjuvant (FCA), whereas for the second injection the antigen was emulsified in an equal volume of Freund's Incomplete Adjuvant (FICA).

Injections. Two injections of P. knowlesi antigen, or PBS in adjuvant were administered intramuscularly in the thigh at one month intervals.

Challenge. The H strain of P. knowlesi was used for challenge. It was maintained in liquid N₂ from where it was passaged into a normal Rhesus monkey. When a patent infection was observed, blood was removed and diluted in sterile physiological saline. All vaccinated animals were injected intravenously with $2.5 \times 10^5$ infected red blood cells one month after the second vaccination. Untreated control animals and adjuvant controls were challenged at the same time.

Hematological studies. Thin smears of peripheral blood were examined daily after being stained with Giemsa stain, and the percent parasitemia was calculated. Red blood cell counts were obtained by use of a Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida).

RESULTS

After challenge with P. knowlesi all monkeys which were not vaccinated with plasmodial antigen died of a fulminating infection. The average day of death of these monkeys was 8.4 (Table 1). Considering all of the vaccinated monkeys as a single group, 8 of 14 survived. The 6 monkeys that died showed some indication of protection as the average day of death for these animals was 14.0, which is significantly later ($p < .005$) than the average day of death of the controls.
Surviving vaccinated monkeys all showed parasites in their peripheral blood. The average peak parasitemia in these monkeys (2.4%) was significantly lower (p < .005) than in the control monkeys (26.3%). The maximum parasitemias of individual monkeys are shown in Table 2. The peak parasitemia in those animals that died may have been artificially low as it represents the last recorded parasitemia in the 24 hours before death and not the actual parasitemia at the time of death.

The prepatent period (interval between infection and detection of parasites in the peripheral blood) was significantly shorter (p < .005) in control animals (3.9 days) than in vaccinated animals (7.0 days). The greatest delay of the prepatent period occurred in the group vaccinated with 500 μg of lyophilized antigen/animal in which the average prepatent period was 10.0 days.

On day 10, all of the control animals had died, whereas 13 of 14 vaccinated animals were still alive. By day 19 all vaccinated animals that were to succumb to the infection had died. After 30 days, at which time the last hematological observations were made, all red blood cell counts had returned to normal and the surviving monkeys had not shown any parasites in their peripheral blood for at least 7 days. Minimum red blood cell counts are shown in Table 2. As in previous experiments there was a substantial drop in the red blood cell counts in many of the protected monkeys.

Three of 4 (75%) monkeys receiving non-lyophilized antigen survived, whereas 5 of 10 (50%) monkeys receiving lyophilized antigen survived. There were not enough animals to determine whether the difference
between the two groups is a real one or whether lyophilized and unlyophilized antigen have a comparable ability to induce protection.

At autopsy it was observed that some monkeys showed tuberculous lesions. This is indicated in Table 2. As far as could be ascertained, these lesions played no role in the protection observed after challenge with *P. knowlesi* as control monkeys with lesions died with a normal fulminating infection.

**DISCUSSION**

The fact that *Plasmodium knowlesi* antigen can be lyophilized, and still retain its protective activity indicates the potential use of such material in tropical areas where storage and shipment of a liquid vaccine would be difficult.

The nature of the protective antigen is as yet uncertain. Electron microscopic studies indicated that the antigen might be parasite membrane. Lyophilization of membranes, however, appears to alter their conformation, and change their immunogenicity by altering immunologically active sites which cannot be restored by rehydration. The protective antigen may be a membrane whose key antigenic determinants are not affected by lyophilization. However, it is possible that protection may be induced by components other than membrane. Analysis in our laboratory of similarly prepared *P. berghei* antigen indicates the presence of non-membrane components, demonstrating the complexity of the vaccinating material. Although the amount of antigen injected was measured on the basis of protein concentration this does not imply that the protective antigen is necessarily a protein, but does suggest that relatively small quantities of antigen can induce protection.
Schenkel et al.\textsuperscript{1} and Simpson et al.\textsuperscript{2} reported immunization of Rhesus monkeys against \textit{P. knowlesi} with French Press prepared antigen and fractionation products. That success and the results of the present study are dependent on incorporation of the antigen in Freund's Complete Adjuvant (FCA). It has not been possible to protect monkeys with antigen alone, or in combination with other adjuvants so far tested. Contrary to work with rodent malaria, FCA alone does not appear to have any effect on \textit{P. knowlesi} in Rhesus monkeys.\textsuperscript{9} The necessity for incorporating the antigen in FCA suggests that cell mediated immunity (CMI) is an important component of the protective response. Further support for this concept is presented by Cabrera et al.\textsuperscript{10} who found a correlation between protection and delayed-type skin reactions in monkeys vaccinated with lyophilized antigen. If CMI is an important component of the protective response it may necessitate the development of an adjuvant which is suitable for large scale use in man, and which also stimulates CMI.

Protection against malaria has also been achieved using attenuated sporozoites as the vaccinating material. Nussenzweig et al.\textsuperscript{11} demonstrated that repeated injections with irradiated \textit{P. berghei} sporozoites protects rodents against a subsequent sporozoite challenge. Successful protection with sporozoite material in rodent malaria has also been reported by others.\textsuperscript{12-14} A recent study suggested that protection can be obtained against \textit{P. falciparum} in volunteers who are fed on by irradiated infected mosquitoes and later challenged by normal infected mosquitoes. However, only one of three such volunteers was protected.\textsuperscript{15} Attempts to vaccinate simians by use of irradiated sporozoites have met with limited success\textsuperscript{16} or have been unsuccessful.\textsuperscript{17}
The demonstration that sporozoites and blood stage antigens can independently induce protection has led to the suggestion that a polyvalent malarial vaccine containing both sporozoite and blood stage antigens may insure a better level of protection than either antigen alone. Optimally, cells primed by vaccination with the blood stage component of a polyvalent vaccine would respond should any sporozoite escape destruction and commence exoerythrocytic development.

It has been found that vaccination with irradiated sporozoites provided protection against sporozoite challenge, but not against challenge with infected erythrocytes.\(^{18}\) We have not yet determined whether vaccination with blood stage antigen provides protection against sporozoite challenge. The demonstration of such protection may cause a re-evaluation of the importance of a polyvalent vaccine.

Upon autopsy several of the monkeys, as noted, demonstrated tuberculous lesions. A recent report\(^{19}\) has focused on the possible protective affect of *Mycobacterium tuberculosis* on subsequent plasmodial infection. We did not observe any differences in the parameters examined between those monkeys that showed tuberculous lesions and those monkeys in which lesions were not observed. Subsequent experiments (Schenkel, unpublished data) further confirm the efficacy of lyophilized antigen.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. R. Jost for technical assistance, and Dr. P. Day and Dr. B. Snyder for maintaining the monkeys used in this study.
REFERENCES


<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of Animals/Group</th>
<th>First day of patency Avg. (range)</th>
<th>Animals Surviving/Animals in Group Days After Challenge</th>
<th>Day of Death Avg. (range)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Non-injected</td>
<td>4</td>
<td>4 (-)</td>
<td>0/4 0/4 0/4 0/4</td>
<td>8.8 (7-10)</td>
</tr>
<tr>
<td>2</td>
<td>Adjuvant + PBS</td>
<td>4</td>
<td>3.8 (3-4)</td>
<td>0/4 0/4 0/4 0/4</td>
<td>8.0 (7-9)</td>
</tr>
<tr>
<td>3</td>
<td>250 µg lyophilized antigen/injection</td>
<td>3</td>
<td>6.3 (5-7)</td>
<td>3/3 2/3 2/3 2/3</td>
<td>11 (-)</td>
</tr>
<tr>
<td>4</td>
<td>500 µg lyophilized antigen/injection</td>
<td>3</td>
<td>10.0 (8-13)</td>
<td>3/3 3/3 1/3 1/3</td>
<td>18 (17-19)</td>
</tr>
<tr>
<td>5</td>
<td>1500 µg lyophilized antigen/injection</td>
<td>4</td>
<td>5.7 (5-7)</td>
<td>3/4 2/4 2/4 2/4</td>
<td>11 (10-12)</td>
</tr>
<tr>
<td>6</td>
<td>1000 µg frozen antigen/injection</td>
<td>4</td>
<td>6.7 (6-8)</td>
<td>4/4 3/4 3/4 3/4</td>
<td>15 (-)</td>
</tr>
<tr>
<td></td>
<td><strong>Summary</strong></td>
<td><strong>8</strong></td>
<td><strong>3.9</strong></td>
<td><strong>0/8 0/8 0/8 0/8</strong></td>
<td><strong>8.4</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Vaccinated</strong></td>
<td><strong>14</strong></td>
<td><strong>7.0</strong></td>
<td><strong>13/14 10/14 8/14 8/14</strong></td>
<td><strong>14.0</strong></td>
</tr>
</tbody>
</table>
Table 2. Parasitemias and red blood cell counts after challenge of vaccinated and control monkeys with *Plasmodium knowlesi*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Monkey Number</th>
<th>State of Health</th>
<th>Outcome after Challenge</th>
<th>Peak % Parasitemia</th>
<th>Day of Challenge</th>
<th>Lowest Reached</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>1</td>
<td>+</td>
<td>D</td>
<td>29.3</td>
<td>5.84</td>
<td>4.46*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>D</td>
<td>19.0</td>
<td>5.41</td>
<td>3.56**</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>D</td>
<td>6.0</td>
<td>5.92</td>
<td>4.33**</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>D</td>
<td>40.0</td>
<td>5.52</td>
<td>3.88***</td>
</tr>
<tr>
<td>Freund's Adjuvant</td>
<td>5</td>
<td>-</td>
<td>D</td>
<td>40.0</td>
<td>5.45</td>
<td>4.18**</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>+</td>
<td>D</td>
<td>11.0</td>
<td>6.14</td>
<td>4.70**</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>-</td>
<td>D</td>
<td>40.0</td>
<td>5.90</td>
<td>4.83**</td>
</tr>
<tr>
<td>Controls</td>
<td>8</td>
<td>-</td>
<td>D</td>
<td>25.2</td>
<td>5.15</td>
<td>3.83*</td>
</tr>
<tr>
<td>Immunized with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 µg Lyophilized Ag</td>
<td>9</td>
<td>-</td>
<td>S</td>
<td>2.2</td>
<td>5.42</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
<td>S</td>
<td>1.1</td>
<td>5.40</td>
<td>3.03</td>
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<tr>
<td>2 x's + Freund's</td>
<td>11</td>
<td>+</td>
<td>D</td>
<td>20.0</td>
<td>5.63</td>
<td>4.74***</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Monkey Number</th>
<th>State of Health</th>
<th>Outcome after Challenge</th>
<th>Peak % Parasitemia</th>
<th>Day of Challenge</th>
<th>Lowest Reached</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunized with 500 µg Lyophilized Ag</td>
<td>12</td>
<td>-</td>
<td>D</td>
<td>14.5</td>
<td>5.53</td>
<td>4.57***</td>
</tr>
<tr>
<td>2 x's + Freund's</td>
<td>13</td>
<td>+</td>
<td>D</td>
<td>5.0</td>
<td>5.97</td>
<td>4.38***</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>14</td>
<td>+</td>
<td>S</td>
<td>0.9</td>
<td>5.34</td>
<td>2.63</td>
</tr>
<tr>
<td>Immunized with 1500 µg Lyophilized Ag</td>
<td>15</td>
<td>+</td>
<td>S</td>
<td>2.8</td>
<td>5.63</td>
<td>2.85</td>
</tr>
<tr>
<td>2 x's + Freund's</td>
<td>16</td>
<td>-</td>
<td>S</td>
<td>&lt;.5</td>
<td>5.63</td>
<td>4.48</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>17</td>
<td>-</td>
<td>D</td>
<td>47.0</td>
<td>5.28</td>
<td>2.76*</td>
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<tr>
<td>Immunized with 1000 µg Frozen Ag</td>
<td>19</td>
<td>-</td>
<td>S</td>
<td>3.4</td>
<td>5.68</td>
<td>3.01</td>
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<tr>
<td>2 x's + Freund's</td>
<td>20</td>
<td>-</td>
<td>S</td>
<td>&lt;.5</td>
<td>6.23</td>
<td>4.36</td>
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<tr>
<td>Adjuvant</td>
<td>21</td>
<td>+</td>
<td>S</td>
<td>8.2</td>
<td>5.89</td>
<td>2.57</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>-</td>
<td>D</td>
<td>21.0</td>
<td>4.88</td>
<td>3.83***</td>
</tr>
</tbody>
</table>

Abbreviations:  + = Evidence of tuberculous lesion  
- = No tuberculous lesion  
D = Dead  
S = Survivor  
* Blood count taken on the day of death  
** Blood count taken one day before death  
*** Blood count taken 2-4 days before death