IDENTIFICATION OF PROTECTIVE COMPONENTS OF TWO MAJOR OUTER MEMBRANE PROTEINS OF SPOTTED FEVER GROUP RICKETTSIAE


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Abstract. Fragments representing the genes of the two major outer membrane proteins of spotted fever group rickettsiae (rOmpA and rOmpB) were tested as DNA vaccines. Immunizations with each of three fragments (rompA<sub>1999–6710</sub>, rompB<sub>1550–2738</sub>, and rompB<sub>4999–6710</sub>) conferred a degree of protection on vaccinated mice against virulent rickettsial challenge. Protection was achieved when DNA immunizations were followed by booster immunizations with the homologous recombinant protein. Proliferation and gamma-interferon secretion were detected after in vitro stimulation of lymphocytes from immunized animals with whole Rickettsia conorii antigen. The data validate particular segments of rOmpA and rOmpB as potent immunogens and hence as sources of immunostimulatory elements with specificity for T lymphocytes, which are the key effectors of protective immunity against rickettsial infections.

INTRODUCTION

Rickettsiae of the spotted fever group (SFG) are obligately intracellular bacteria that are transmitted by the bite of infected ticks or mites. Immediately after injection via the arthropod saliva into the skin, rickettsiae grow initially in undetermined cell types in the bite site and apparently spread via the lymphatic vessels to regional lymph nodes where they multiply prior to spreading to the blood stream and infecting the endothelium throughout the body. Infection of the endothelium causes vascular damage leading to increased vascular permeability, edema, hypovolemia and hypotension. Both humoral and T cell mediated immune responses follow infection. After recovery, immunity is solid and long lasting, no human reinfections have been reported, and experimental animals that have recovered from SFG rickettsioses are solidly immune to rechallenge. The mechanisms of protective immunity, however, are incompletely understood. T cell-mediated responses are considered more crucial than humoral immunity. CD8<sup>+</sup> T lymphocytes play an important role in the ultimate immune clearance of rickettsiae from the endothelium. The intracellular killing process is known to be mediated in mice by gamma-interferon (IFN-γ), tumor necrosis factor-alpha, and nitric oxide.

Currently, there is no effective vaccine against any SFG rickettsia, and despite the availability of improved and more sensitive immune assays for detecting rickettsial infection, no human reinfections have been reported, and experimental animals that have recovered from SFG rickettsioses are solidly immune to rechallenge. The mechanisms of protective immunity, however, are incompletely understood. T cell-mediated responses are considered more crucial than humoral immunity. CD8<sup>+</sup> T lymphocytes play an important role in the ultimate immune clearance of rickettsiae from the endothelium. The intracellular killing process is known to be mediated in mice by gamma-interferon (IFN-γ), tumor necrosis factor-alpha, and nitric oxide.

It is encoded as a 168-kD protein that includes a putative β-autotransporter and undergoes post-translational processing to its mature 135-kD form. rOmpB of the typhus group rickettsiae has been referred to as a surface protein antigen (SPA) and is thought to form part of an S-layer. rOmpA and rOmpB elicit both humoral and T cell-mediated immune responses. The majority of monoclonal antibodies from mice inoculated with SFG rickettsiae react with heat-stable or heat-labile epitopes present on these two proteins or with the cell wall lipopolysaccharide.

In addition, T cell clones have been developed against epitopes of each protein, indicating the presence of both CD8 and CD4 epitopes (Feng HM, Walker DH, unpublished data). Immunization with recombinant rOmpA of R. rickettsii protects guinea pigs solidly against a lethal dose of R. rickettsii. Moreover, immunization with recombinant rOmpA of R. conorii completely protects guinea pigs against challenge with R. conorii and partially protects against challenge with R. rickettsii.

All these findings support rOmpA and rOmpB as potential vaccine candidates; however, a better candidate would be a vaccine that contains only the antigens that strongly stimulate the arms of the immune system that are relevant for the clearance of rickettsial infections, particularly CD8 and CD4 T lymphocytes. Thus, the objective of this study was the

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identification of the T lymphocyte immunostimulatory fragments present in rOmpA and rOmpB that confer protection against a lethal challenge with R. conorii. Three fragments, rompA\(1200-1710\), rompB\(1550-2738\), and rompB\(2459-4123\), were able to stimulate some degree of protection against virulent rickettsial challenge. Protection was better achieved when DNA immunizations were followed by booster immunizations with the homologous recombinant protein.

**MATERIALS AND METHODS**

*Rickettsia. R. conorii* (Malish 7 strain), a human isolate from South Africa, was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultivated in specific pathogen-free embryonated chicken eggs. Rickettsial suspensions were prepared by homogenization of infected yolk sacs using a Waring blender and diluted to a 10% suspension in sucrose-phosphate-gluamate buffer (SPG; 218 mM sucrose, 3.8 mM KH\(_2\)PO\(_4\), 7.2 mM K\(_2\)HPO\(_4\), 4.9 mM monosodium L-glutamic acid, pH 7.0). Aliquots of the stock were kept frozen at −80°C. Particular rickettsial antigen was prepared by renografin density gradient purification from infected Vero cells (African green monkey kidney cells; ATCC). After purification, rickettsiae were killed by exposure to 100°C for 10 min.

**Plasmid DNA constructs.** rOmpA fragments were amplified by a polymerase chain reaction (PCR) from the plasmid vector pBA13 (Table 1). pBA13, a generous gift from Robert Gilmore, was used in this system. Proteins are expressed as a fusion protein containing the homologous recombinant protein.

**Table 1**

<table>
<thead>
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<th>Fragment</th>
<th>Size (kb)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>5′-ATGCGGTGGACAGACAGGAATTT-3′</td>
<td>5′-GATACCGATGCTCCAAGAG-3′</td>
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</table>

**Protein purification.** Recombinant proteins encoded by each one of the rickettsial gene fragments were prepared. Two plasmid expression systems were used. The first one used the pGEX system (Amersham Pharmacia Biotech, Piscataway, NJ). The desired DNA fragments were generated by restriction enzyme digestion and cloned into the pGEX plasmid backbone. Transformation into competent *E. coli* BL21 cells was carried out following standard procedures. Correct frame and orientation were confirmed by automated DNA sequence analysis. Single colonies of transformed *E. coli* were picked and grown in 2× YT-G medium (1.6% tryptone, 1% yeast extract, 0.085 M NaCl, and 2% glucose, pH 7.0) with 100 μg/ml of ampicillin. Expression was induced using 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the culture reached an absorbance at 260 nm \(A_{600}\) of 1.5–2.0. After induction, cells were incubated at 37°C for 4 hr. Subsequently, the cells were pelleted and lysed by freezing/thawing and sonication. The lysate was incubated with glutathione sepharose beads. Recombinant glutathione-S-transferase (GST)-fusion rickettsial proteins bound to the glutathione beads. The beads were washed thoroughly to eliminate other contaminant proteins and were kept at 4°C in phosphate-buffered saline until used for immunization. The levels and quality of expression obtained using this system varied from preparation to preparation. In addition, GST controls showed high background stimulation of lymphocyte proliferation in some *in vitro* antigen-stimulation experiments. For these reasons, the pCR\(\beta\)/CT-TOPO \(\beta\) (Invitrogen) expression system was used subsequently. This system uses the same cloning strategies as the mammalian expression vector pCDNA3.1/V5-His-TOPO \(\beta\) (Invitrogen). With this system, proteins are expressed as a fusion protein containing six consecutive histidine residues (6×His tag). After transformation, frame and orientation were confirmed by automated DNA sequencing analysis. Plasmids were maintained as *E. coli* TOP10 F transformants. For expression and purification of recombinant proteins, competent *E. coli* BL21 cells were transformed with each plasmid construct. The culture was incubated overnight in a volume of 10 ml under ampicillin selection (100 μg/ml), and 500 ml of fresh LB medium containing 100 μg/ml of ampicillin were inoculated with the overnight culture. The culture was incubated with vigorous shaking until reaching an \(A_{600}\) of 1.5–2.0. IPTG (0.1 mM) was added to induce expression. Flasks were incubated for an additional 4 hr. After induction, cells were pelleted and kept frozen at −80°C overnight. The pellet was thawed for 15 min and resuspended in lysis buffer (100 mM NaH\(_2\)PO\(_4\), 10 mM Tris-Cl, 8 M urea, pH 8.0). The solution was stirred for 1 hr at room temperature until it became translucent. The lysate was centrifuged at 10,000 × g for 30 min at room temperature to pellet cellular debris, and the
supernatant fluid was collected. Proteins were purified using an Ni-NTA spin kit (Qiagen). This system uses nickel-nitro-

loacetic acid (Ni-NTA) metal affinity chromatography ma-
trices for proteins that contain a 6×His tag. Purification was
carried out according to the manufacturer’s instructions. Re-
combinant proteins were separated in a sodium dodecyl sul-
fate–polyacrylamide gel, transferred to a nylon membrane
and detected with an anti-6×His antibody conjugated to
alkaline phosphatase (Invitrogen).

**Mice, immunizations, and challenge.** The maintenance
and care of experimental animals complied with the National
Institutes of Health guidelines for the humane use of labo-

ratory animals. Three sets of immunization experiments
were carried out (Table 2). Six to eight-week-old male C3H/

HeN mice (Harlley Sprague-Dawley, Indianapolis, IN) were
used in all three experiments. Mice were provided unrestricted
access to food and water. During the first experiment, animals
received three immunizations with 100 µg of plasmid DNA mixed
with 100 µg of pWRG/mIL-12 (pIL-12). Plasmid interleukin-12 (pIL-12) is a pBluescript® plasmid
(Stratagene, La Jolla, CA) that encodes the two subunits of
murine interleukin-12, p40 and p35. Both subunits are under
separate CMV promoter regions and bovine growth hormone
poly A signal. pIL-12 was kindly provided by Dr. Hua Yu
(Moffitt Cancer Center, Tampa, FL). The first two DNA im-
munizations were administered intramuscularly in the tibialis
anterior muscles four weeks apart. A third DNA immuni-

zation was administered subcutaneously using a Biojector
(Bioject, Inc., Portland, OR). A Biojector is a needle-free
device that uses compressed CO2 to inject fluids. Half of the
mice that received DNA immunizations also received two
subcutaneous booster immunizations of the homologous pu-

rified recombinant protein (100 µg) each four weeks apart.
Control groups included mice vaccinated with non-recom-
binant vector, non-recombinant vector plus GST, saline only,
or a sublethal dose of *R. conorii*.

For the second experiment, animals were immunized with
only two doses of DNA (100 µg of recombinant plasmid
and 100 µg of pIL-12) four weeks apart given intramuscu-
larly in the tibialis anterior muscles. An additional fragment
(rompB2176–3933) that was not available for the first experiment
was included. All mice that received DNA immunizations
were also administered two booster immunizations of 100
µg each of the homologous purified recombinant protein.
Three groups that received only the two immunizations with
one of the three recombinant proteins of *rOmpA* or *rOmpB*
(without DNA vaccination) were included in this experi-
ment. Control mice received either non-recombinant vector plus
β-galactosidase–His recombinant protein (negative control)
or a sublethal immunizing dose of *R. conorii* (positive control).

During the third experiment, animals received a mixture
of four plasmids containing the fragments *rompA*2176–3933
*rompB*2176–3933 and *rompA*4999–6710. The fragment *rompA*4999–6710
was also included. Half of the mice immunized with the
DNA mixture received two booster immunizations contain-
ing a mixture of the homologous recombinant proteins. A
group that received only the recombinant protein mixture
was included. Positive and negative control groups were the
same as in experiment two. All mice immunized with re-
combinant protein preparations received 300 µg of the anti-

histaminic drug Promethazine® (Elkins-Sinn, Cherry Hill,
NJ) subcutaneously prior to the challenge to prevent an an-

aphylactic response to the inoculum.

Mice were challenged with 3 median lethal doses (LD50)
of *R. conorii*. In a previous experiment, this dose was shown
to kill 100% of naïve mice. After challenge, animals were
observed daily for morbidity and mortality. Data were ana-
lyzed using Fisher’s exact test. Differences between data sets
were termed significant if *P* < 0.05.

**Lymphocyte responses.** T lymphocyte responses were
determined by measurement of cell proliferation and IFN-γ
secretion after antigen stimulation. The standard thymidine
incorporation assay was used to quantify lymphocyte prolif-
eration. Briefly, spleens were aseptically removed and sepa-
rated into single cell suspensions. Splenocytes were treated
with a solution containing 0.15 M ammonium chloride, 1
mM KHCO3, and 0.1 mM Na2EDTA, pH 7.2–7.4 to lyse the
erythrocytes. Lymphocyte suspensions were adjusted to a
concentration of 1 × 106 cells/ml. One hundred microliters
per well were aliquoted into 96-well plates. Purified partic-
ulate *R. conorii* antigen was added at concentrations of 1,
0.3, and 0.1 µg/ml to triplicate wells. Plates were incubated
at 37°C in an atmosphere containing 5% CO2. On day 5, 1

### Table 2

*Immunization regimens*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Immunization regimen</th>
<th>Recombinant peptide</th>
<th>Other</th>
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<td>R. conorii</td>
</tr>
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</table>

**Immunization regimens**

- **Experimen**
- **Immunization regimen**
- **Recombinant peptide**
- **Other**
μCi of "H-thymidine was added per well, and the plates were incubated overnight at 37°C. Cells were harvested into GF/B (Packard Instruments Co., Meriden, CT) plates. After evaporation to dryness, 30 μl of scintillation fluid was added to each well. "H-thymidine incorporation was determined in a beta counter (Top Count, Packard Instruments Co.). Lymphocyte proliferation was considered significant if the proliferation index (cpm of sample stimulated with antigen/cpm of sample without antigen) was greater than 3. IFN-γ secretion was measured using a quantitative sandwich enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). Supernatants of splenocytes (5 × 10^6/ml per well in a 24-well plate) were collected after stimulation with purified particulate R. conorii antigen for 72 hr. IFN-γ concentration was determined following the manufacturer’s instructions. Results reported are the average of triplicate samples. Standard deviations were determined using Microsoft (Redmond, WA) Excel. Differences among data sets were considered significant if P ≤ 0.05; P values were determined using Student’s t-test with Microsoft Excel. Antibody production was determined by indirect immunofluorescence assay.

RESULTS

Cloning and expression of rickettsial fragments. DNA fragments representing the two major cell wall proteins of R. rickettsii (Figure 1) were cloned into prokaryotic and mammalian expression vectors. Results are reported only for the fragments that conferred some degree of protection after challenge with a lethal dose of R. conorii. These fragments were rompA_4999–6710, rompB_1550–2738, and rompB_2459–4123. Homologous recombinant proteins (rOmpA_1644–2213, rOmpB_451–846, and rOmpB_754–1308, respectively) were prepared using the pCR®/CT-TOPO vector (Invitrogen). Recombinant rickettsial proteins were detected by Western immunoblot using an antibody directed against the fusion His tag (Figure 2).

Lymphocyte response. During the first set of experiments, spleens were harvested one day before challenge, and splenocytes were stimulated with three different doses of purified particulate R. conorii antigen. Splenocytes from mice that received only DNA vaccinations containing the fragment rompA_4999–6710 did not show higher levels of cell proliferation than the control groups (Figure 3). Only after the mice were boosted with the homologous recombinant protein RompB_1550–2738 did the proliferation index reach higher levels than the controls. Mice that received DNA vaccine containing the fragment rompB_2459–4123 showed higher levels of lymphocyte proliferation than the controls. These levels

![Figure 1](image1.png)  
**Figure 1.** Rickettsia rickettsii fragments representing the genes encoding rOmpA and rOmpB were amplified by a polymerase chain reaction and cloned into mammalian and prokaryotic expression vectors. All fragments were tested as plasmid DNA vaccines. After immunization, four fragments (filled blocks) were shown to confer some degree of protection after a challenge with a lethal dose of R. conorii.

![Figure 2](image2.png)  
**Figure 2.** Western immunoblot of the expressed and purified recombinant His tag fusion proteins of rOmpA_1644–2213 (lane 1), rOmpB_451–846 (lane 2), and rOmpB_754–1308 (lane 3) encoded by rompA_4999–6710, rompB_1550–2738, and rompB_2459–4123, respectively, reacted with anti-His antibody. Molecular mass markers are indicated in lane M. kDa = kilodaltons.

![Figure 3](image3.png)  
**Figure 3.** Lymphocyte proliferative response after antigen stimulation of splenocytes from mice immunized with DNA vaccine preparations alone or with DNA vaccine preparations followed by booster immunizations with the homologous recombinant protein. Splenocytes from mice that received both DNA and recombinant protein preparations proliferated after antigen stimulation. Splenocytes from mice that received only DNA vaccine preparations containing the fragment rompA_4999–6710 did not show higher levels of cell proliferation than the control groups (Figure 3). Only after the mice were boosted with the homologous recombinant protein RompB_1550–2738 did the proliferation index reach higher levels than the controls. Mice that received DNA vaccine containing the fragment rompB_2459–4123 showed higher levels of lymphocyte proliferation than the controls. These levels
PROTECTIVE ANTIGENS OF SFG RICKETTSIAE

Figure 4. Gamma interferon (IFN-γ) secretion after in vitro stimulation of splenocytes from mice immunized with DNA vaccines alone or boosted with recombinant proteins with whole Rickettsia conorii particulate antigen. Secretion of IFN-γ correlated with cell proliferation. None of the splenocytes that were not stimulated with R. conorii antigen secreted IFN-γ.

Figure 5. Gamma interferon (IFN-γ) secretion after Rickettsia conorii antigen stimulation of splenocytes from mice immunized with DNA preparations only (A). Two mice were studied per group. Each bar represents the response of an individual animal. After receiving two DNA immunizations, mice were boosted twice with the homologous recombinant protein. Lymphocyte-stimulated IFN-γ secretion was measured after the second protein immunization (B). Groups of mice immunized twice with recombinant proteins only were evaluated along with mice immunized with DNA vaccines and boosted with recombinant proteins. Splenocytes from two mice per group were pooled and stimulated with R. conorii antigen in vitro. Significantly higher levels of IFN-γ (P < 0.05) were detected in the supernatants of the splenocytes of all the immunized mice.

During the second set of experiments, spleens were harvested for antigen stimulation studies at two time points during the course of the experiment. The first time point was after the second DNA immunization. IFN-γ levels in the supernatants of these splenocytes cultured in the presence of R. conorii antigen were higher (P ≤ 0.05) than the controls for all fragments except for one of the two mice immunized with the DNA fragment rompB2499–4123 (Figure 5A). The second time point was one day before challenge with live R. conorii; at this point mice had received either both DNA and recombinant protein preparations or only recombinant protein. IFN-γ secretion by antigen-stimulated lymphocytes was observed not only when mice received both DNA and recombinant protein but also when they had only received recombinant protein immunizations (Figure 5B). For fragment rompA4999–6710, the IFN-γ levels were higher after DNA immunization followed by booster immunization with recombinant protein. For the other two fragments, rompB1550–2739 and rompB2499–4123, the IFN-γ levels were similar in animals immunized with the recombinant proteins regardless of receiving DNA immunizations or not. These results suggest that immunization with plasmids containing these two fragments did not enhance the T lymphocyte reactivity against rickettsiae.

Evidence of seroconversion was not observed during the course of any of the experiments except for the animals that received a sublethal dose of R. conorii.

Protective immunity. During the first set of experiments, mice received either three doses of DNA or three doses of DNA followed by two doses of homologous recombinant protein. Immunizations were carried out four weeks apart. Four weeks after the last immunization, animals were challenged with 3 LD50 of R. conorii. Mice were observed daily for morbidity and mortality. Mice in all groups except for the positive immunity control group (convalescent from a...
sublethal dose of *R. conorii* started to show signs of illness by day 4 post-inoculation. Mortality was observed on days 6 through 8. All mice that survived until day 9 recovered. Protection of all three mice per group was observed only when animals were immunized with DNA and boosted with the homologous recombinant protein and differed significantly (*P* = 0.05) from both unvaccinated and animals vaccinated with DNA only. Immunization with only DNA preparations did not confer any protection (Table 3). The second experiment, mice were immunized with DNA and boosted with the corresponding recombinant proteins. In addition, groups of mice that received only recombinant protein immunizations were included. The results (Table 4) corroborated our findings that protection was achieved when DNA immunizations are followed by booster immunizations with the homologous recombinant protein. Protection was also observed in animals that received only recombinant protein preparations. Although the percentage of survivors among the mice that received only recombinant protein preparations was lower (14–43%) than in the groups that received both DNA and recombinant protein preparations (60%), the differences were not statistically significant possibly because of the small number of animals in each group. Experiment three showed that indeed the combination of DNA and recombinant protein preparations conferred better protection than protein immunizations alone (37.5% versus 100%; *P* = 0.028). Total protection (100%) was observed after DNA immunization with a mixture of the identified protective DNA fragments followed by booster immunizations containing a mixture of the corresponding recombinant proteins.

## DISCUSSION

The development of vaccination strategies against intracellular parasites offers special challenges to scientists, and rickettsiologists are no exception. Despite the difficulties associated with their growth, preparation, and containment and the small number of scientists engaged in their study, three vaccine candidates have been developed against SFG rickettsioses. However, none of them have been proven to be sufficiently effective for current use. The reasons for their failure remain unknown and clearly reveal the need for more studies with focus on the mechanisms involved in the protective immune responses. It is known that cell mediated responses are the key effectors. Thus, an effective vaccine will be one that contains the immunostimulatory elements required to activate such types of responses. The vaccine will have to be designed in a way that ensures effective processing and presentation of epitopes to T lymphocytes by host cells. Until recently, live attenuated vaccines were the most effective approach to the development of protective vaccines, but safety issues, especially for pregnant women and immunocompromised hosts, remain a concern. The development of nucleic acid immunizations as vaccine strategies offers an attractive alternative for efficient antigen processing, presentation and priming of T lymphocytes. In addition to being safe, stable at room temperature and inexpensive to prepare, DNA vaccines are amenable to genetic manipulation, making it possible to include only the appropriate immunostimulatory sequences in a single vaccine preparation. Since the first demonstration of DNA vaccination as an efficient method of generating cytotoxic T lymphocyte activity and antibody secretion against the nuclear protein of influenza virus A, numerous studies of infectious diseases and cancer using plasmid DNA immunization have been reported.39,40

The novel strategy for vaccination known as “prime-boost” (priming with DNA vaccines followed by boosting with attenuated recombinant viral vectors expressing the relevant antigens) has been shown to produce increased specific immune responses against the influenza virus, malaria, and human immunodeficiency virus.31-45 In this study, the prime-boost protocol used involved DNA vaccination followed by boosting with the corresponding recombinant peptide. This approach has been proven effective only in systems in which

### Table 3

Protective immunity after DNA or DNA and recombinant protein immunizations

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<th>Mice per group (n)</th>
<th>Morbidity (onset of illness)</th>
<th>Survival</th>
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<td>rompB&lt;sub&gt;2459-4123&lt;/sub&gt;</td>
<td>3 (4–5)</td>
<td>0/3</td>
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<tr>
<td>rompB&lt;sub&gt;2459-4123&lt;/sub&gt; + rompB&lt;sub&gt;374-1308&lt;/sub&gt;</td>
<td>3 (4–5)</td>
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<tr>
<td>pcDNA + pIL-12</td>
<td>3 (4–5)</td>
<td>0/3</td>
</tr>
<tr>
<td>pcDNA + pIL-12 + GST</td>
<td>3 (4–5)</td>
<td>1/3†</td>
</tr>
<tr>
<td><em>Rickettsia conorii</em></td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td>7</td>
<td>7 (4–5)</td>
</tr>
</tbody>
</table>

* There were no survivors among mice that were immunized with the DNA vaccines containing rompA<sub>1644-2213</sub> (0/3), rompA<sub>1644-2213</sub> (0/3), or rompB<sub>374-1308</sub> (0/3) nor mice immunized with rompA<sub>1644-2213</sub> and recombinant protein rompA<sub>1644-2213</sub> (0/3).

† Survivor showed severe permanent neurologic damage.

### Table 4

Protective immunity after DNA and recombinant protein or recombinant protein only immunizations

<table>
<thead>
<tr>
<th>Mice per group (n)</th>
<th>Morbidity (onset of illness)</th>
<th>Survival</th>
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<tbody>
<tr>
<td>rompA&lt;sub&gt;499-6710&lt;/sub&gt; + rompA&lt;sub&gt;1644-2213&lt;/sub&gt;</td>
<td>5 (4–5)</td>
<td>3/5</td>
</tr>
<tr>
<td>rompB&lt;sub&gt;1550-2739&lt;/sub&gt; + rompB&lt;sub&gt;451-644&lt;/sub&gt;</td>
<td>5 (4–5)</td>
<td>3/5</td>
</tr>
<tr>
<td>rompB&lt;sub&gt;451-644&lt;/sub&gt;</td>
<td>7 (4–5)</td>
<td>2/7</td>
</tr>
<tr>
<td>rompB&lt;sub&gt;2459-4123&lt;/sub&gt; + rompB&lt;sub&gt;374-1308&lt;/sub&gt;</td>
<td>5 (4–5)</td>
<td>3/5</td>
</tr>
<tr>
<td>pcDNA + pIL-12 + rHis</td>
<td>5 (4–5)</td>
<td>0/5</td>
</tr>
<tr>
<td><em>Rickettsia conorii</em></td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 5

Protective immunity after immunization with quadrivalent preparations of DNA, recombinant protein, or both

<table>
<thead>
<tr>
<th>Quadrivalent vaccine preparations</th>
<th>Mice per group (n)</th>
<th>Morbidity (onset of illness)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA*</td>
<td>6</td>
<td>6 (4–5)</td>
<td>2/6</td>
</tr>
<tr>
<td>Recombinant protein†</td>
<td>8</td>
<td>8 (4–5)</td>
<td>3/8</td>
</tr>
<tr>
<td>Plasmid DNA and recombinant protein‡</td>
<td>6</td>
<td>6 (4–5)</td>
<td>6/6</td>
</tr>
<tr>
<td>pcDNA + pIL-12 + rHis</td>
<td>6</td>
<td>6 (4–5)</td>
<td>1/6</td>
</tr>
<tr>
<td><em>Rickettsia conorii</em></td>
<td>6</td>
<td>0</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* rompA<sub>1644-2213</sub> + rompA<sub>2176-3933</sub> + rompA<sub>451-644</sub> + rompA<sub>1550-2739</sub>
† rompA<sub>499-6710</sub> + rompA<sub>2176-3933</sub> + rompB<sub>2459-4123</sub> + rompB<sub>1550-2739</sub>
‡ rompA<sub>499-6710</sub> + rompA<sub>2176-3933</sub> + rompB<sub>2459-4123</sub> + rompB<sub>1550-2739</sub> + rompA<sub>1644-2213</sub> + rompB<sub>374-1308</sub> + pcDNA + rIL-12 + rHis + *Rickettsia conorii*
humoral responses play a crucial role. In this study, protection was achieved in the absence of antibodies after prime-boost using recombinant peptides.

During this study, we exploited DNA vaccine technology in an attempt to identify the immunostimulatory components of the two major outer proteins of SFG rickettsiae, rOmpA and rOmpB. As mentioned earlier, rickettsiae are intracellular organisms, and this condition presents different challenges for the immune system. Like most intracellular infections, rickettsioses are best controlled by mechanisms mediated by T lymphocytes with antibody playing a secondary role. Both rOmpA and rOmpB contain B and T lymphocyte epitopes and, thus, represent good candidates for a vaccine. In this study, one fragment from rOmpA and two fragments from rOmpB conferred protection against challenge with a lethal dose of R. conorii. Protection appeared to be better achieved among the groups that received both DNA and recombinant protein immunizations, although recombinant protein immunizations alone provided some protection. This situation suggests the presence of CD4 epitopes and an important role for CD4 T lymphocytes, since soluble proteins do not effectively enter the MHC Class I presentation pathway.

Total protection (100% of mice) was observed when vaccine was administered as a multivalent preparation of the protective DNA fragments and the corresponding recombinant proteins. The mixture was composed of the fragments described in this study and a fourth fragment, rOmpA\textsubscript{1576-1915}. Fragment rOmpA\textsubscript{1576-1915} encodes the last six repeat units of Domain II followed by 368 basepairs of Domain III. (Figure 1). Protection can be achieved after plasmid DNA immunization with this fragment followed by a booster immunization with recombinant protein or by immunization with recombinant Mycobacterium vaccae organisms expressing the fragment followed by immunizations with the homologous recombinant protein (Crocquet-Valdes PA and others, unpublished data).

The fact that protection was achieved in the absence of detectable antibody corroborates the findings that humoral responses play a less important role in protective immunity. This result may be due to a Th1-biased response due to the coexpression of IL-12; therefore, the potential importance of B cell epitopes of rOmpA and rOmpB should not be discarded.

This study represents the first report of the use of recombinant plasmid DNA as vaccine strategy against rickettsial infections. The levels of protection reported are encouraging; more experiments intended to optimize the expression of the fragments and priming of T lymphocytes are currently taking place. These include the identification of CD8 T-lymphocyte epitopes at the oligopeptide level and the exploration of more effective means of antigen availability for processing and presentation.

In addition, the data presented represent the first reported evidence that rOmpB is an immunoprotective antigen of a SFG rickettsia and identify portions of rOmpA and rOmpB that stimulate protective immunity.

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