Yersinia pestis, the causative agent of bubonic plague and pneumonic plague, is a gram-negative pathogen that infects many animal species, including humans, and is transmitted by arthropod vectors or aerosol droplets (16). Immunization with purified recombinant LcrV (rLcrV) is sufficient to generate protective immunity to both bubonic plague and pneumonic plague in mice, guinea pigs, and non-human primates (6, 7, 10, 12–14, 24, 25, 28). Brubaker and colleagues showed that LcrV injection of animals triggered release of interleukin-10 (13), a cytokine that suppresses innate immune functions (21). rLcrV also prevents the release of proinflammatory cytokines (gamma interferon and tumor necrosis factor) (15) in murine and human macrophages (2, 12, 13). Considering the immune modulatory properties of rLcrV, there are concerns regarding the safety of rLcrV vaccines in humans (15).

We searched for variants with reduced immune modulatory properties (15). rV10, a variant lacking amino acids 271 to 300 of LcrV, displayed a significant decrease in its ability to induce interleukin-10 and to suppress tumor necrosis factor alpha or gamma interferon release (15). Immunization of mice with rV10 protects against lethal plague infections caused by 1,000 mean lethal doses (MLD) of Y. pestis KIM5 (KIM D27) (15), a Δpgm (pigmentation defective), attenuated strain that causes plague in mice, guinea pigs, and non-human primates (6, 7, 10, 12–14, 24, 25, 28). Brubaker and colleagues showed that LcrV injection of animals triggered release of interleukin-10 (13), a cytokine that suppresses innate immune functions (21). rLcrV also prevents the release of proinflammatory cytokines (gamma interferon and tumor necrosis factor alpha) in murine and human macrophages (2, 12, 13). Considering the immune modulatory properties of rLcrV, there are concerns regarding the safety of rLcrV vaccines in humans (15).

In contrast to Yersinia pestis LcrV, the recombinant V10 (rV10) variant (lacking residues 271 to 300) does not suppress the release of proinflammatory cytokines by immune cells. Immunization with rV10 generates robust antibody responses that protect mice against bubonic plague and pneumonic plague, suggesting that rV10 may serve as an improved plague vaccine.
succumbed to disease within 4 days after infection with an average time-to-death of 2.5 days (Fig. 1 and Table 1). These data demonstrate that, similar to rLcrV, rV10 immunization of mice provides robust protection against bubonic plague infections.

Pneumonic plague infections in mice can be precipitated via aerosol inhalation or intranasal infection. Aerosol infection of mice is technically demanding and requires high doses of *Y. pestis* (1). To develop an intranasal infection model of *Y. pestis* CO92, groups of 10 BALB/c mice were infected with bacterial suspensions delivered by the intranasal route. Actual deposition in the lungs was determined by postmortem removal of lungs 60 min after inoculation, followed by plating of tissue homogenate and colony formation. After infection with $1.9 \times 10^5$ CFU of *Y. pestis* CO92, greater than 80% of inoculated bacteria were found deposited in lung tissues. Animals were monitored for 14 days for signs of lethal disease or death and time-to-death was recorded. An average dose of 389 CFU (MLD) caused lethal disease in half of all experimental animals, consistent with previous observations using *Y. pestis* bio-

### TABLE 1. Vaccine protection elicited by rV10 and rLcrV immunization against intranasal challenge with *Y. pestis* CO92

<table>
<thead>
<tr>
<th>Inoculum (CFU)</th>
<th>Alhydrogel</th>
<th>rLcrV</th>
<th>rV10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4.5 \times 10^7$</td>
<td>9/9 (2.1)</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td>$6.7 \times 10^7$</td>
<td>NT</td>
<td>8/9 (3.0)</td>
<td>9/9</td>
</tr>
<tr>
<td>$5.6 \times 10^8$</td>
<td>NT</td>
<td>7/9 (3.0)</td>
<td>9/9</td>
</tr>
<tr>
<td>$4.0 \times 10^9$</td>
<td>NT</td>
<td>2/9 (2.4 ± 1.2)</td>
<td>3/9 (4.2 ± 2.5)</td>
</tr>
</tbody>
</table>

* Anesthetized BALB/c mice were infected by intranasal inoculation and observed for 14 days for the development of plague. 

### FIG. 1. Vaccination of mice with rV10 provides protection against bubonic plague. BALB/c mice were immunized intramuscularly with adjuvant alone (Alhydrogel), rLcrV, or rV10 in a two-dose regimen (50 μg of purified, endotoxin-free antigen injected on day 0 and 21). On day 43 postimmunization, mice were challenged with 100,000 MLD of *Y. pestis* CO92 by subcutaneous injection, and survival was monitored.

### FIG. 2. Vaccination of mice with rV10 provides protection against pneumonic plague. (A) BALB/c mice were immunized intramuscularly by following the standard two-dose regimen with adjuvant alone, rLcrV, or rV10. On day 43 postimmunization, mice were challenged with 1,000,000 CFU (equivalent to 2,570 MLD) of *Y. pestis* CO92 via intranasal instillation, and survival was monitored. (B) rLcrV-specific IgG1, IgG2A, and IgG2B antibodies were measured by ELISA in sera of five animals immunized with either rLcrV or rV10, rV10 immunization generated significantly higher titers for IgG1 ($P < 0.001$), IgG2A ($P < 0.0181$), and IgG2B ($P < 0.0064$). Statistical significance of differences in IgG titers was interrogated with a paired Student’s *t* test, and *P* values were recorded.

var Medievalis strain KIM (26). The average time to death varied, depending on dose, with high-dose animals succumbing to infection on day 2, while animals infected with lower doses developed lethal infections 4 days after inoculation. Groups of 10 BALB/c mice were immunized with rLcrV or rV10 according to the two-dose regimen described above. On day 43 after primary immunization, mice were challenged with 2,570 MLD of *Y. pestis* CO92 (1,000,000 CFU) by intranasal inoculation. rLcrV vaccination provided 70% protection in this experiment, whereas mice immunized with rV10 were completely protected (Fig. 2). These data suggest that rV10 vaccination is at least as efficacious against lethal pneumonic plague challenge as rLcrV immunization. Sera collected from immunized mice on day 42...
after primary immunization were analyzed by enzyme-linked immunosorbent assay (ELISA) for total immunoglobulin G (IgG) specific for rLcrV or rV10. The data revealed a significant increase in anti-rLcrV IgG antibody titer in rV10-vaccinated mice compared with rLcrV-immunized animals (1.3 × 10^5 [± 8.2 × 10^3] for rV10 and 2.5 × 10^4 [± 0] for rLcrV; P < 0.001, determined with a Student’s t test).

To compare rV10 and rLcrV vaccine efficacy, we investigated breakthrough challenges in mice immunized with adjuvant, rLcrV, or rV10. On day 43 after immunization, animals...
were infected intranasally with doses ranging from \(4.5 \times 10^7\) to \(4.0 \times 10^8\) CFU. Adjuvant control mice succumbed to disease. Mice vaccinated with rLcrV or rV10 were fully protected at a challenge dose of \(4.5 \times 10^7\) CFU. Mice immunized with rV10 and challenged with \(6.7 \times 10^6\) or \(5.6 \times 10^7\) CFU were also fully protected; however, mice immunized with rLcrV were only partially protected as 1/9 and 2/9 animals, respectively, succumbed to infection. At the highest dose, \(4.0 \times 10^8\) CFU, rV10 offered partial protection as 6/9 mice succumbed to infection, whereas rLcrV vaccination protected only 2/9 mice. Thus, in comparison with rLcrV, rV10 vaccination offered at least equal levels of plague vaccine protection.

rLcrV vaccination generates antibodies that provide protection against plague by improving the efficiency of host polymorphonuclear cell phagocytosis of \(Y.\) pestis and by blocking bacterial type III injection of effector proteins into host cells (18, 27). Immune sera from BALB/c mice vaccinated with either rLcrV or rV10 were characterized for the immunoglobulin type III injection of effector proteins into host cells. pMM83 encodes YopM-Bla, a hybrid between the YopM effector with a C-terminal fusion to β-lactamase (9). CCF2-AM is a membrane-permeant ester with two fluorophores attached to cephalosporin that exhibit fluorescence resonance energy transfer. Excitation of coumarin (409 nm) results in green fluorescence emission that rLcrV-immunized mice harbored a significant proportion of blue cells (1.77% of splenocytes). As a control, adjuvant mice infected with glutathione transferase (GST)-Bla \(Y.\) pestis harbored no blue cells. One mouse that had been vaccinated with LcrV harbored \(Y.\) pestis KIM5 (pMM83) in the spleen, and 1% of its splenocytes stained blue. No blue cells could be found in the other two rLcrV-immunized mice that did not harbor bacteria in the spleen. None of the rV10-vaccinated mice harbored blue cells or were infected with \(Y.\) pestis, suggesting that rV10 vaccination efficiently clears the infection (Fig. 3).

In sum, using bubonic plague and pneumonic plague models, we observed high levels of protection afforded by rV10 immunization in mice that correlated with the development of specific humoral and cellular immune responses that are at least equivalent to those raised with rLcrV. As rV10 displays reduced immune modulatory properties, this antigen may serve as a safe and effective subunit vaccine for humans. To test this prediction, rV10 vaccine efficacy and safety will need to be examined in non-human primates with aerosol challenge of \(Y.\) pestis CO92 as a measure for protection against pneumonic plague.

The authors acknowledge membership within and support from the Region V “Great Lakes” Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Consortium (NIH Award 1-U54-AI-057153).

REFERENCES
enterocolitica type III secretion, is required for toxin targeting into the cytoplasm of HeLa cells. J. Biol. Chem. 275:36869–36875.


Editor: V. J. DiRita