Further development of raccoon poxvirus-vectored vaccines against plague (*Yersinia pestis*)

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ABSTRACT

In previous studies, we demonstrated protection against plague in mice and prairie dogs using a raccoon pox (RCN) virus-vectored vaccine that expressed the F1 capsular antigen of *Yersinia pestis*. In order to improve vaccine efficacy, we have now constructed additional RCN-plague vaccines containing two different forms of the *lcrV* (V) gene, including full-length (Vfull) and a truncated form (V307). Mouse challenge studies with *Y. pestis* strain CO92 showed that vaccination with a combination of RCN-F1 and the truncated V construct (RCN-V307) provided the greatest improvement ($P = 0.01$) in protection against plague over vaccination with RCN-F1 alone. This effect was mediated primarily by anti-F1 and anti-V antibodies and both contributed independently to increased survival of vaccinated mice.

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1. Introduction

Plague, caused by *Yersinia pestis*, is a zoonotic disease transmitted primarily by fleas that has recently re-emerged in numerous parts of the world. To reduce this public health threat and to protect against the potential use of *Y. pestis* as a bioweapon, development of novel plague vaccines for both humans and animals has been the focus of extensive research in recent years [1–3]. The capsular F1 antigen (17.5 kDa) and the secreted V antigen (35 kDa) are natural virulence factors produced by *Y. pestis* [4,5] and have been shown to be highly immunogenic, conferring a degree of protection equivalent to that of live plague vaccines such as the EV76 vaccine [6]. Vaccines based on these two antigens and a recombinant protein fusion of F1 and V have been developed [7], providing distinct advantages in comparison to the previous bacterial vaccines. However, like most proteins, they are usually weakly immunogenic when administered in the absence of an appropriate adjuvant or when administered via the oral route.

As an alternative, we have developed plague vaccines using raccoon poxvirus (RCN) as a vector for *Y. pestis* antigens. Raccoon poxvirus was first isolated from the upper respiratory tract tissue of 2 of 92 apparently healthy raccoons captured in 1961–1962 during a survey of wildlife at Aberdeen Proving Grounds, Maryland [8]. Like vaccinia and canary poxviruses, RCN has been used previously as an experimental vaccine vector [9]. A significant advantage of RCN compared to other poxvirus vectors approved for veterinary applications is its ability to induce immune responses when delivered by mucosal routes [10]. Recombinant RCN (rRCN) vaccines have been administered to a variety of mammalian species, including mice, rats, rabbits, raccoons, skunks, bobcats, cats, dogs, and sheep [9–12], prairie dogs [13] and black-footed ferrets (Rocke, unpublished data) with no reported side effects. rRCN vaccines have induced protective immune responses against rabies in raccoons, dogs, cotton rats, rabbits, bobcats, and foxes [9,11,14], and protective immune responses in domestic cats against feline panleukopenia virus, feline caliciviruses, and feline infectious peritonitis [12,15]. In a previous study, we demonstrated that a vaccine with several molecular elements, such as the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) and tissue plasminogen activator (tPA), significantly enhanced the expression levels of F1 in a recombinant RCN-based vaccine [16]. This vaccine, designated RCN-F1, was shown to fully protect mice against intradermal (i.d.) challenge with *Y. pestis* [16] at challenge doses as high as 4.8×10^3 LD$_{50}$. The RCN-F1 vaccine was also efficacious in prairie

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dogs vaccinated orally via voluntary consumption of vaccine-laden baits [13,17].

For this study, we designed two additional RCN-vectored vaccines containing different forms of the low calcium response V gene (*lcrV*) in an attempt to improve vaccine efficacy and to potentially provide protection against F1 negative strains of *Y. pestis*. Because the LcrV (or V) antigen has been associated by others with the suppression of gamma interferon and tumor necrosis factor alpha *in vivo* [18,19], we constructed a truncated form (V307) of the *lcrV* gene, removing the purportedly immunosuppressive sequences [20]. Both this and a full-length *lcrV* gene (V full) were then inserted into our RCN vector and tested in mice in combination with RCN-F1 via simultaneous injections. Serological analyses and animal challenge studies revealed that vaccination of mice with RCN-F1 in combination with the truncated V construct (RCN-V307) provided the greatest protection when compared with RCN-F1 in combination with RCN-Vfull or RCN-F1 alone.

2. Materials and methods

2.1. Cells and viruses

Rat embryonic fibroblasts [Rat-2 (ATCC #CRL-1764)] and African green monkey kidney epithelial cells [BSC-1 (ATCC #CCL-26) and Vero (ATCC#CCL-18)] were maintained at 37 °C and 5% CO_2 in Medium 199 supplemented with 0.01 g/L L-glutamine and 5% fetal bovine serum (FBS) and were used for culturing virus. Raccoon poxvirus (RCN) Herman strain [8] was mixed 1:1 with trypsin–versene solution (0.05% trypsin; 0.02% EDTA in Earle's Balanced Salt Solution) and incubated for 15 min at 37 °C to release infectious particles from aggregates that may have formed upon storage prior to inoculation into cells.

2.2. Construction of pTK transfer vectors

Construction of RCN-F1 was described previously [16]. For RCN-V constructs, various versions of the *Y. pestis lcrV* gene were cloned into the polylinker region of the pTK shuttle vector [16] so that the introduced DNA and upstream *cis*-acting elements (the p11 late promoter of vaccinia virus and the EMCV-IRES) were flanked by RCN thymidine kinase gene (*tk*) sequences. The introduced genes (lacking their native translation initiation codons) were inserted in-frame behind the tPA secretory signal sequence (aa 2–23). Two versions of *lcrV* were each PCR-amplified and separately cloned into the pTK vector to construct (1) pTK-tPA-Vfull (RCN-Vfull), expressing the full-length (326 aa) antigen (PCR primers: forward 5′-CATATGATTAGAGCCTACGAA-3′; reverse 5′-GGATCCTCATTTACCAGACGT-3′) and (2) pTK-tPA-Vt$_{307}$ (RCN-V307), expressing a 307-aa C-terminally truncated V antigen (PCR primers: forward 5′-GGCGCCGGCATTAGAGCCTACGAACAA-3′; reverse 5′-GCGGATCCTCAACGGTTCAGTGCTTC-3′).

2.3. Generation of recombinant RCN constructs

The pTK shuttle vectors described above were used to deliver each version (full-length or truncated) of the *Y. pestis lcrV* gene into the RCN genome through *tk*-driven homologous recombination. The process of generating *tk*-disrupted RCN recombinants (rRCN) has previously been described in detail [16]. Briefly, BSC-1 cells at 80% confluence were infected at a multiplicity of infection (MOI) of 0.06 with wild type RCN. Infected cells were then transfected in serum-free Opti-MEM (Invitrogen, Carlsbad, CA) with a mixture containing 4 μg of plasmid DNA and 10 μL of lipofectamine 2000 (Invitrogen, Carlsbad, CA) per well of a 6-well plate, according to the instructions provided by the manufacturer. The cell medium was replaced 5 h later. Three days post-infection/transfection, cells were freeze-thawed three times and the lysate was re-plated on Rat-2 cells in M199 medium containing 5% FCS and 50 μg/ml 5-bromo-2-deoxyuridine (BrdU). Following a 2 h incubation, the medium was removed and the infected monolayer overlaid with 1X MEM containing 5% FCS and 1.2% low melt agarose. In the presence of BrdU, only *tk*− rRCN give rise to plaque forming units (pfu). Individual BrdU-resistant plaques were picked approximately 14 days post-inoculation (PI) and passaged twice through Rat-2 cells grown in BrdU-containing medium. Purified viral clones were then amplified for large scale purification in Vero cells as described previously [16].

To evaluate if our RCN-vectored V antigens were similar to V antigens produced by *E. coli* clones, Vero cells at confluence in 75 cm² flaks were infected with 0.1 MOI of RCN-Vfull and RCN-V307 until cytopathic effect was observed. Cells and supernatants were collected, frozen and thawed 3 times and kept at −80 °C until use. After denaturation of the lysate (20 μl) at 100 °C for 10 min in a buffer 0.5%SDS, 40 mM DTT, N-deglycosylation was then performed using PNGase F enzyme (New England Biolabs, Ipswich, MA) accordingly to manufacturer instructions: 1000 units of PNGase F and 1% NP-40 added to denatured lysates for 1 h at 37 °C. In parallel, Vfull purified protein [provided by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID)] and V307 purified protein produced in *E. coli* [ORIGAMI^{TM} B(DE3)placI cells (Novagen, EMD Chemicals Inc., Gibbstown, NJ)] using the plasmid pTriEx-4 Neo (Novagen) were denatured and N-deglycosylated using the same protocol.

2.4. In vitro expression of rRCN constructs

The *in vitro* expression of *Y. pestis* LcrV antigens by our rRCN constructs, as well as the deglycosylated proteins, was determined by Western blot as described previously [16], using polyclonal mouse anti-LcrV antiserum and goat-anti-mouse secondary antibody conjugated to alkaline-phosphatase obtained from KPL, Inc. (Gaithersburg, MD).

2.5. Animal studies

Four animal studies were conducted with the rRCN/*Y. pestis* vaccine constructs. The first study was designed to determine the most effective RCN-V vaccine construct. Unfortunately, due to an unavoidable delay in the experiment, older mice (18-week-old A/J mice obtained from Harlan Sprague Dawley) were used in this experiment compared to those described below. Groups of 12 mice each received the vaccine formulations singly or combined as listed in Table 1. All rRCN constructs were inoculated intramuscularly (IM) in the thigh with a volume of 0.2 ml. Animals that received two rRCN constructs were inoculated with each virus in separate thighs. RCN-F1 was inoculated at a dosage level of 10^7 pfu (the level shown to be protective in previous studies), whereas the RCN-V constructs were inoculated at a dosage level of 10^8 pfu. A group of 11 mice received the empty vector virus (RCN-TK−) and was used as a negative control. All animals were boosted (same formulations, dosage, and route) on day 28 post-initial inoculation. Four weeks after the boost, all animals were challenged with the CO92 isolate of *Y. pestis* (provided by USAMRIID). Each group of 12 mice was subdivided into groups of animals and each received either 7×10^4 cfu (3.5×10^3 LD50), 7×10^5 cfu (3.5×10^4 LD50), or 7×10^6 cfu (3.5×10^5 LD50) of *Y. pestis*. Stock aliquots of the bacteria were prepared and quantified as previously described [16], and diluted in sterile saline. A volume of 0.2 ml of each dilution was administered to each mouse by i.d. injection in the abdominal region. Plate counts of the challenge inoculum confirmed the dose administered and concurrent mouse tests confirmed its virulence. The mice were monitored for 24 days for signs of illness

Table 1
Vaccine treatments and dosages administered to A/J mice via intramuscular inoculation and their response to challenge with *Yersinia pestis* (CO92) at various dosages. (ND = not done). Median survival time (MST) is the day by which 50% of animals had succumbed to infection.

Expt.#	n	Vaccine	Dosages (pfu)	% survival to *Y. pestis* challenge (MST)		
				70,000 cfu	700,000 cfu	7,000,000 cfu
1	12	RCN-TK⁻	1×10^7	0 (3)	0 (2)	0 (2)
1	12	RCN-F1	1×10^7	0 (8)	25 (12)	0 (2)
1	12	RCN-F1+RCN-V307	1×10^7, 1×10^8	100	75	25 (2)
1	12	RCN-F1+RCN-Vfull	1×10^7, 1×10^8	50 (11)	50 (13)	25 (3)
2	8	RCN-TK⁻	1×10^7	0 (2)	ND	0 (2)
2	8	RCN-F1	1×10^7	100	ND	0 (3)
2	8	RCN-V307	1×10^7	0 (2)	ND	0 (2)
2	8	RCN-F1+RCN-V307	1×10^7, 1×10^7	75	ND	25 (4)
3	8	RCN-TK⁻	1×10^7, 1×10^7	ND	0 (2)	ND
3	8	RCN-F1+RCN-TK−	1×10^7, 1×10^8	ND	25 (4)	ND
3	8	RCN-F1+RCN-V307	1×10^7, 1×10^8	ND	50 (8)	ND
3	8	RCN-F1+RCN-V307	1×10^7, 5×10^7	ND	63	ND
3	8	RCN-F1+RCN-V307	5×10^7, 5×10^7	ND	89	ND
4	4	RCN-TK⁻	5×10^7	ND	0 (4)	ND
4	8	RCN-F1	5×10^7	ND	50 (14)	ND
4	8	RCN-V307	5×10^7	ND	0 (3)	ND
4	8	RCN-Vfull	5×10^7	ND	13 (3)	ND
4	8	RCN-F1+RCN-TK−	5×10^7, 5×10^7	ND	63	ND
4	8	RCN-F1+RCN-V307	5×10^7, 5×10^7	ND	89	ND
4	8	RCN-F1+RCN-Vfull	5×10^7, 5×10^7	ND	75	ND

or death. Animals with obvious clinical signs (labored breathing, severe lethargy) were humanely euthanized as were all survivors at the end of the 24-day post-infection period.

The second and third experiments were designed to determine the most effective vaccine dosages. Groups of 6–8-week-old A/J mice (8–12 mice per group) were vaccinated IM with the vaccine combinations listed in Table 1. One group of mice in each experiment received RCN-TK⁻ and served as the negative control. The animals were boosted with the same formulations and dosages on day 28 PI. Four weeks after the boost, the animals were challenged with *Y. pestis* via i.d. injection and monitored for morbidity and mortality for 21 days as described above.

A fourth experiment was conducted to confirm results of the previous experiments. Groups of 6–8-week-old A/J mice (8 mice per group) were immunized and boosted as described above with single (RCN-F1, RCN-Vfull, RCN-V307) or combined (RCN-F1+RCN-Vfull, RCN-F1+RCN-V307, RCN-F1+RCN-TK⁻) vaccines. A control group of 4 mice received RCN-TK⁻ only. All constructs were inoculated at a dosage level of 5×10^7 pfu, the level that gave the best protection in experiment 3.

Plague-induced mortality was verified in selected mice in each experiment by isolation of *Y. pestis* specific DNA sequences from bacterial culture via PCR. Frozen carcasses were thawed and necropsied, and liver samples were plated on blood agar plates (Becton-Dickinson, Franklin Lakes, NJ) at 28 °C for up to 72 h. Suspect colonies were cultured in brain heart infusion broth (Difco, Location). The DNA was subsequently extracted from the culture broth and stored at −20 °C. For PCR, primers specific for the *Y. pestis* F1 gene [21] were used to amplify DNA fragments that were fractionated and directly visualized using standard techniques.

2.6. Serology

Blood samples (50 μl) were collected from the medial saphenous vein of each mouse at the times of initial vaccination, boost, and challenge; blood samples were also obtained from all survivors at study termination. Serum was collected and stored at −20 °C.

Antibody titers to *Y. pestis* F1 and V were determined using ELISA with antigens supplied by USAMRIID as described previously [16]. Serum samples were serially diluted 4-fold from 1:160 to 1:163,840 and test samples were run in duplicate. Titers of 1:160

were considered negative. The highest dilution that was positive (exceeded the mean of four negative control samples by three standard deviations) was considered the endpoint and its reciprocal value recorded as the titer.

2.7. Statistical analyses

All analyses were performed with SAS statistical software (SAS Institute Inc., Cary NC). The effects of treatments on survival rates were examined with the Cox proportional hazards model, which takes into account time to death as well as survival rates. We used the stratified version of the Cox model, with experiment as the stratifying variable. This model assumes that the relative effects of the treatments remain the same between experiments, but the model permits the baseline survivorship to vary freely. Antibody titers were transformed by calculating the $\log_{10}$ of the titer in order to normalize the data and reduce skew. The effect of treatments on the log antibody titers were determined using analysis of variance with additive treatment and experiment effects. To adjust for experiment effects, the SAS least squares means (population marginal means) for the treatments and pair-wise significance tests for each of them were computed using the SAS PDIFF option. For data display, we present the least squares means along with ±2 standard errors computed from the treatment groups pooled across experiments. We also analyzed each experiment individually for verification of the combined analysis; because the results were consistent, we report only those for the combined analysis.

3. Results

3.1. In vitro expression of V antigen by RCN viruses

Two RCN-vectored vaccines were constructed containing either the full-length *lcrV* (RCN-Vfull) gene or a truncated form (RCN-V307). That particular truncation was chosen because it was the location of a convenient restriction site (Xho I) and previous studies by Overheim et al. [20] showed a similar truncation (at amino acid 301) reduced the immunosuppressive properties of the LcrV protein without compromising protection against plague challenge in mice. The *in vitro* expression of RCN-Vfull and RCN-V307 vaccines was examined by western blot analyses at 24 and 48 h PI (Fig. 1). As expected from our previous studies on RCN-F1 [16],
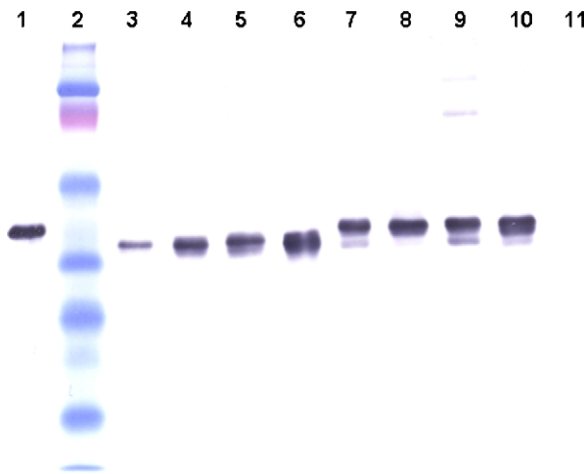
Fig. 1. Western blot of RCN-V307 and RCN-Vfull infected vero cell monolayers. The pellet or supernatant was subjected to PAGE (5% stacking, 12% resolving, 200 V, 1 h). Samples were transferred to a nitrocellulose membrane (30 V, overnight), and blocked in TBST 5% dry-milk overnight. The membrane was probed in 1:1000 mouse anti-V serum for 1 h, washed, and alkaline-phosphatase-conjugated goat-anti-mouse secondary antibody (Promega) and BCIP/NBT color development substrate was used to visualize V antigen. Lanes are: (1) V protein positive control; (2) prestained protein molecular weight markers 19–108 kDa (Cedarlane Laboratories); (3) V307 24 h cell pellet; (4) V307 24 h cell supernatant; (5) V307 48 h cell pellet; (6) V307 48 h cell supernatant; (7) Vfull 24 h cell pellet; (8) Vfull 24 h cell supernatant; (9) Vfull 48 h cell pellet; (10) Vfull 48 h cell supernatant (11) RCN-Tk 48 h whole. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the use of the EMCV-IRES and the tPA secretory signal resulted in strong expression levels of the V307 and Vfull antigens in the cell pellet and supernatant of Vero-infected cells, both at 24 and 48 h PI. No difference was detected in the migration between proteins from the RCN-V constructs, their deglycosylated counterparts, and the equivalent proteins derived from *E. coli* (data not shown), suggesting that the V antigens expressed by the RCN-V constructs were similar in size and conformation to the purified proteins.

3.2. Animal studies

To evaluate the efficacy and immunogenicity of our RCN-V constructs, we conducted a series of sequential experiments and analyzed the data with statistical procedures that allowed us to compare the combined results. The proportion of animals that survived plague challenge in each treatment group and the median survival time (MST) for those groups with ≤50% survival are included in Table 1. In every case, all the control animals that received the empty vector (TK$^-$) died from plague challenge within 3–4 days PI. Interestingly, nearly all of the animals that received either RCN-Vfull or RCN-V307 alone did not survive challenge either. In contrast animals that received RCN-V constructs in combination with RCN-F1 had better survival rates. Using a Cox proportional hazards model, stratified by experiment, we first compared survival of all treatment groups against survival of groups that received RCN-F1 alone at a dosage of 1×10^7 pfu. Although all the mice that received RCN-F1 in experiment 1 unexpectedly died at the low challenge dose on day 8, this statistical model incorporates mean time to survival, as well as survival rates, over all challenge doses and experiments, and removing this group did not significantly alter the results. All vaccinated groups with a hazard ratio (HR) < 1.0 relative to RCN-F1 (1×10^7 pfu) are included in Fig. 2. There was no difference in the hazard ratio (HR = 0.27; P = 0.42) between RCN-F1 administered at 1×10^7 pfu or 5×10^7 pfu, nor did the addition of RCN-TK$^-$ at either dosage significantly alter protection (HR = 0.46; P = 0.27 and HR = 0.20; P = 0.33). The biggest improvement over RCN-F1 alone was obtained when this construct was administered in combination with RCN-V307 at dosages of 5×10^7 pfu (HR = 0.06, P = 0.01). With this treatment, 89% of mice were protected following challenge with 700,000 cfu of *Y. pestis*. The next best treatment was 1×10^7 pfu RCN-F1 and 1×10^8 pfu RCN-V307 (HR = 0.25; P = 0.01). The combination of RCN-F1 and RCN-Vfull at either of the dosages tested did not significantly improve survival (P > 0.10) of plague-challenge mice relative to RCN-F1 alone.

We also performed a Cox proportional hazards analysis that included anti-F1 and anti-V titers in addition to all of the treatment groups. The hazard ratios were 0.50 (P = 0.006) and 0.59 (P = 0.006) respectively, indicating that increased survival was associated with
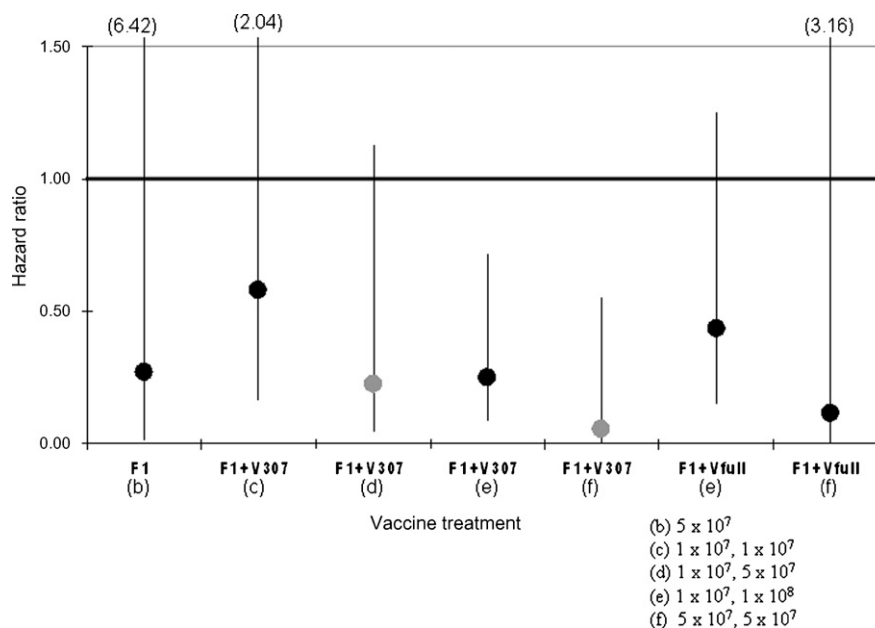

Fig. 2. Hazard ratio and 95% confidence intervals for rRCN vaccine treatments administered to mice i.m. either singly or in combination in relation to RCN-F1 (at a dosage of 1×10^7). Letters in parenthesis indicate the dosages administered.
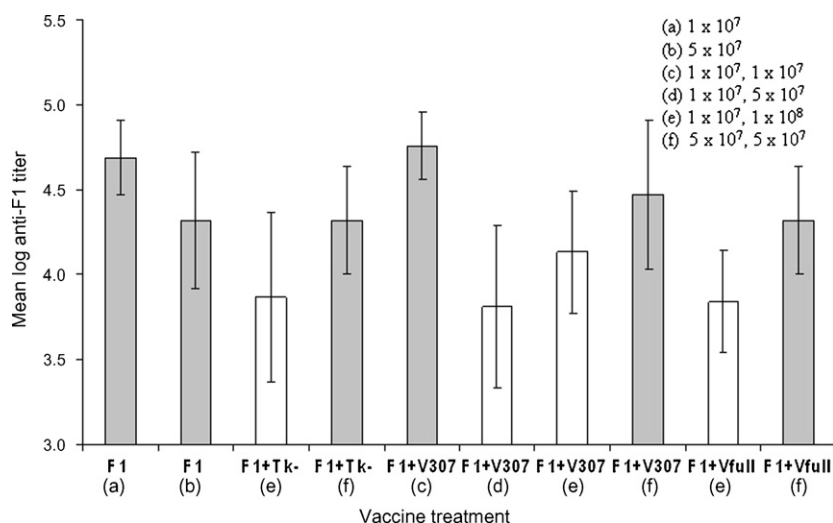
Fig. 3. Least squares mean log anti-F1 titer for rRCN vaccine treatments administered to mice i.m. either singly or in combination. Letters indicate the dosages administered. Error bars represent ±2 standard errors computed from the treatment groups pooled across experiments. Gray bars indicate treatments that are not significantly different ($P > 0.05$) from each other, but that are significantly higher ($P < 0.05$) than treatments indicated by white bars.

both increased anti-F1 and anti-V antibodies. By running the model without treatment effects and with just anti-F1 and anti-V antibodies, we constructed a likelihood ratio statistic for a treatment effect of $G^2 = 14.1$ (d.f. = 14), which is not significant ($P > 0.5$). This result suggests that the treatment effect is primarily modulated through antibody levels. It is worth noting that the hazard ratio for each antibody was adjusted for the presence of the other. The fact that both anti-F1 and anti-V antibodies remain significant when both are present in the model means that they both contribute independently to the survival of the challenge subjects.

Antibody titers were compared between treatment groups using analysis of variance with additive experiment and treatment effects. Antibody titers to F1 were significantly elevated ($P < 0.0001$) in all treatment groups that received RCN-F1 alone or in combination with another RCN construct compared to controls (TK$^-$) and vaccinates that did not receive the RCN-F1 construct (data not shown). Population marginal means for anti-F1 titers of all treatment groups that received RCN-F1 alone or in combination with

another RCN construct are shown in Fig. 3. No difference in mean anti-F1 antibody titer was noted between RCN-F1 administered alone at a dosage of 1×10^7 or 5×10^7 pfu and any of the other groups that received an additional construct (RCN-TK$^-$, RCN-V307, or RCN-Vfull) as long as the construct was administered at an equivalent dosage. In those groups that received RCN-F1 at a dosage of 1×10^7 pfu and a larger dosage of the second construct (5×10^7 or 1×10^8 pfu), anti-F1 titers were significantly lower ($P < 0.005$) than that of RCN-F1 alone. This appears to have resulted from the additional virus and not the V protein as this was observed in groups that received the empty vector (RCN-TK$^-$) as well as RCN-V307 or RCN-Vfull in combination with RCN-F1.

Anti-V antibody titers were significantly elevated ($P < 0.0001$) in all treatment groups that received RCN-Vfull or RCN-V307 alone or in combination with RCN-F1 compared to controls (TK$^-$) and vaccinated groups that received RCN-F1 or RCN-F1+RCN-TK$^-$ (data not shown). Population marginal means for those groups with elevated anti-V titers are shown in Fig. 4. No difference in anti-V titer was
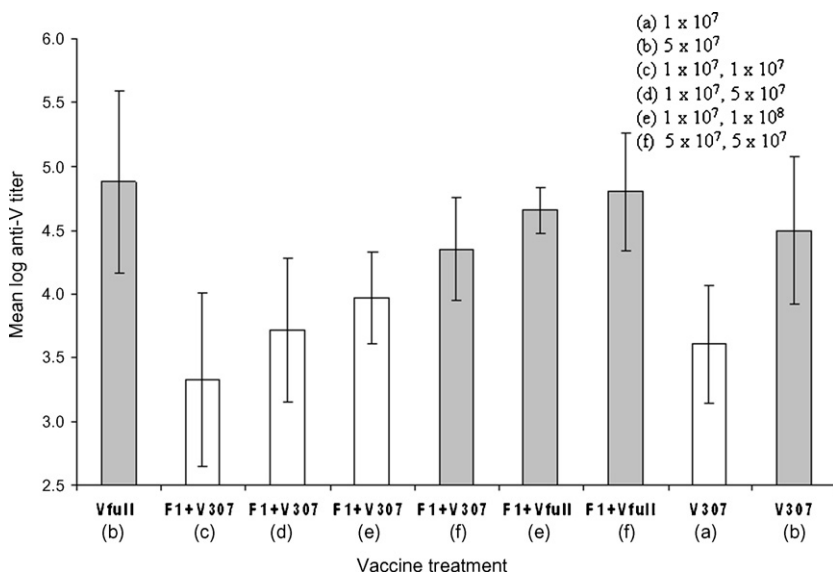

Fig. 4. Least squares mean log anti-V titer for rRCN vaccine treatments administered to mice i.m. either singly or in combination. Letters indicate the dosages administered. Error bars represent ±2 standard errors computed from the treatment groups pooled across experiments. Gray bars indicate treatments that are not significantly different ($P > 0.05$) from each other, but that are significantly higher ($P < 0.05$) than treatments indicated by white bars.

noted between the group that received RCN-Vfull alone and those that received RCN-Vfull in combination with RCN-F1 ($P > 0.05$). Likewise, the anti-V titers of groups that received RCN-V307 at a dosage of 5×10^7 pfu alone or in combination with an equivalent dosage of RCN-F1 were not significantly different ($P > 0.05$). However, groups that received RCN-V307 at a dosage of 1×10^7 pfu or in combination with RCN-F1 at 1×10^7 pfu had a lower mean anti-V titer than the RCN-Vfull group ($P < 0.001$).

4. Discussion

We had previously reported that mice [16] and prairie dogs [13] immunized with a rRCN-vectored vaccine expressing *Y. pestis* F1 antigen (RCN-F1) were protected against plague challenge. At a dosage of 1×10^7 pfu, the RCN-F1 vaccine provided full protection in vaccinated mice upon challenge with 100,000 cfu of *Y. pestis* and partial protection (56%) of orally immunized prairie dogs challenged with 130,000 cfu. In this study, we designed a new vaccine construct expressing a truncated form of the V gene (RCN-V307) that significantly improved the survival of mice ($P = 0.01$) when administered in combination with RCN-F1. The majority (89%) of mice vaccinated with a combination of RCN-F1 and V307 at a dosage of 5×10^7 for each construct survived a plague challenge dose of 700,000 cfu. In contrast, increasing the dosage of RCN-F1 alone to 5×10^7 pfu did not significantly improve survival of plague-challenged mice ($P = 0.42$).

Interestingly, administration of RCN-Vfull in combination with RCN-F1 was not as effective as RCN-V307. As a major virulence factor of *Y. pestis*, full-length LcrV antigen has been shown to trigger the release of interleukin 10 (IL-10) by host immune cells, a cytokine that suppresses innate immune functions [19,22]. The elevated IL-10 suppresses the release of pro-inflammatory cytokines, such as tumor necrosis factor alpha and gamma interferon, altering the defense mechanisms required to combat the pathogenesis of plague [19,22]. This immunosuppressive property of full-length LcrV reduces its desirability as a vaccine candidate, but recently Overheim et al. [20] reported the construction of several LcrV variants and demonstrated their use as recombinant vaccines against plague. In their studies, a truncated LcrV antigen lacking amino acid residues 301–326 (rV11) elicited immune responses that protected mice against a lethal challenge with *Y. pestis*. Compared to full-length LcrV, rV11 (as well as other constructs) displayed a reduced ability to release IL-10 from mouse and human macrophages, enabling an increased response by the innate immune system. The RCN-V307 construct developed in this study contains a similar deletion of 19 aa at the C-terminus of the LcrV gene (308–326). Thus, the increased protective efficacy of the RCN-V307 construct compared to RCN-Vfull may have resulted from removal of the immunosuppressive region located within the truncated sequence. We are currently comparing the cytokine profiles induced by these vaccine constructs.

Even if cytokines play some role in the treatment response, our data analysis suggests that the effect of vaccination was primarily mediated by induction of antibody to F1 and V antigens. Although anti-F1 had more influence than anti-V when both antibodies were present, both appeared to contribute independently to increased survival of challenged mice. The F1 antigen is a capsule-like protein expressed by *Y. pestis* at $37\,^{\circ}$C, after the bacteria begins multiplying in eukaryotic tissues, but not at $28\,^{\circ}$C [23], the approximate body temperature of fleas and the temperature we used to grow our *Y. pestis* challenge inoculum. Early in flea-transmitted plague infection (and presumably in our challenge system), the bacteria is highly sensitive to phagocytosis and is taken up by tissue macrophages and perhaps also epithelial cells [24], where they gain resistance to phagocytosis through the type III secretion system and proteins encoded by the virulence plasmid common to all pathogenic *Yersinia* species (Yops). Once the thermally induced F1 antigen is expressed, it renders the pathogen even more able to resist phagocytocis by preventing binding between *Y. pestis* and phagocytic cells [25]. This resistance to uptake by phagocytes and neutrophils allows the pathogen to rapidly multiply, leading to a lethal systemic infection. Therefore, antibody to F1 in vaccinated mice may reduce the ability of *Y. pestis* to evade phagocytosis upon i.d. inoculation.

Antibody to V antigen in vaccinated mice that also had anti-F1 antibody clearly improved their response to i.d. challenge in our study, perhaps by blocking the immunomodulatory activity of *Y. pestis* LcrV [19], and/or by blocking the delivery of Yops in infected macrophage-like cells [26]. However, nearly all (23/24) of the animals vaccinated with single RCN-V constructs died upon *Y. pestis* challenge, even at our lowest challenge dose of 3500 LD50s (70,000 cfu), suggesting anti-V antibody alone was insufficient to protect the animals. Other studies have demonstrated the suitability of the LcrV antigen as a vaccine against F1+ and F1− *Y. pestis* when delivered as a single subunit protein [27,28] or DNA vaccine [29] at challenge doses as high as 10^7 cfu [30]. In these studies, the subunit vaccines were all delivered in the presence of Alhydrogel, an aluminum adjuvant known to significantly boost antibody response [31], and most studies were conducted in Balb/C or similar mouse breeds. We used A/J mice for our studies, because they are known to be highly susceptible to poxviruses [32], and other mouse models were much less responsive to our RCN vaccine constructs (data not shown). However, we recognize that the enhanced susceptibility of A/J mice to viruses and other agents is due to immune deficiencies, including defective macrophage tumoricidal and lymphocyte proliferative responses [33] and deficiencies in complement reactivity and natural killer cell function [34,35]. It is possible that these immunological deficiencies in A/J mice may have influenced their full immune response to V antigen upon vaccination or to *Y. pestis* upon challenge in those animals that received RCN-V alone. Administration of combined F1 and V constructs appeared to overcome these deficiencies, but further testing will be required to test these hypotheses. Nonetheless, our goal is to develop a safe vaccine that provides protection against plague exposure for ultimate use in free-ranging prairie dogs via oral immunization. The results of this preliminary study in mice suggest that the combination of RCN-F1 and RCN-V307 vaccines would go farthest towards meeting that goal, and in fact, recent studies in prairie dogs confirm this hypothesis [36].

In summary, when administered in combination with RCN-F1, our newly designed RCN-V307 vaccine significantly increased protection against plague in A/J mice. The best survival and highest anti-F1 and anti-V antibody titers in vaccinated mice were derived from a combination of 5×10^7 pfu for each virus, a feasible dosage from a production standpoint. Although a dual injection vaccine is not our desired final product, the results of our study suggest that a combination vaccine vectored by RCN that contains both F1 and V antigens is a better alternative than single vaccines containing F1 or V alone. Because of their ability to induce immune responses following mucosal (oral) vaccination, our rRCN-plague vaccine constructs hold promise for wild and domestic animals. Further work is in progress to test these rRCN-plague constructs in prairie dogs via oral administration, to further evaluate the immune response and mechanism of action in both mice and prairie dogs, and to determine their protective efficacy against other *Y. pestis* strains, including F1 negative strains.

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