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Novel vaccination strategies to prevent or ameliorate pneumonic plague caused by a non-encapsulated strain of *Yersinia pestis*.

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Abstract

Plague is an ancient disease caused by *Yersinia pestis*, a widely disseminated Tier 1 pathogen that poses significant public health and biowarfare risks. The rapid course and high mortality of pneumonic plague limit the efficacy of antibiotic treatment and mandate the need for an effective, licensed, and readily available vaccine. New candidate vaccines are being developed, however their efficacy in nonhuman primates, optimal vaccination schedule and immune response, duration of protection, and breadth of coverage against various virulent strains are inadequately understood. In the current work, we explored homologous and heterologous vaccination schemes using the sensitive BALB/c mouse models of bubonic and pneumonic plague and challenges with *Y. pestis* strain C12. This strain, a derivative of wild type strain CO92, lacks the anti-phagocytic F1 capsule yet remains highly virulent. Protection against such nonencapsulated strains has been particularly elusive. We tested the efficacy of live attenuated vaccine (LAV) derivatives of CO92 or C12 with a deletion of the virulence-associated gene *yscN* or of the *pgm* pigmentation locus and were cured of the pPst (PCP1) plasmid (CO92 *pgm*$^-$ pPst$^-$). The LAVs were evaluated alone or accompanied by a dose of protein subunit vaccine. The most protective and immunogenic vaccination scheme, as tested under a variety of conditions in the two plague models, was heterologous vaccination with an LAV and the recombinant rF1V or rV protein subunit vaccine. Furthermore, in the heterologous scheme, different LAV and subunit vaccines could be substituted, affording flexibility in vaccine component selection. We also evaluated a novel intervention strategy combining vaccination and post-exposure antibiotic treatment. The layering of vaccination and post-exposure treatment with streptomycin was synergistic, extending the time after *Y. pestis* C12 challenge when treatment remained effective and affording a sparing of antibiotic. Thus, the current work defined effective and flexible vaccination and treatment interventions which successfully prevented lethal infection with virulent, nonencapsulated *Y. pestis*.

Introduction

The protection afforded by rF1V against F1-negative strains of *Y. pestis* relies solely on the V antigen component. Since there is evidence for V heterogeneity within *Yersinia* species, the potential exists that naturally occurring or engineered strains harboring altered V antigens could overcome rF1V induced immunity.

Thus, a more effective plague vaccine that can induce strong antibody and cell-mediated immune responses in various animal models of plague is needed. The vaccine efforts in our laboratory are focused on the modification and optimization of our candidate LAVs and defined protein subunit vaccines. The LAV candidates evaluated in this study are highly attenuated due to a deletion in the *yscN* gene, which encodes for an ATPase which provides energy to the T3SS and is necessary for Yop effector translocation.

Here we explored homologous and heterologous vaccination schemes utilizing both the Δ*yscN* mutants as standalone or paired with the rV or rF1V subunit vaccines in the sensitive BALB/c mouse model of protection against aerosol challenge with a F1 capsule-negative *Y. pestis* strain. Optimal protection against challenge with nonencapsulated *Y. pestis* was achieved by heterologous vaccination with a live strain and a protein subunit vaccine. In addition, we initiated studies for a novel intervention strategy layering vaccination and post-exposure antibiotic treatment. Disease outcome improved and a delayed initiation of antibiotics was still protective when vaccination was integrated with post-exposure antibiotic treatment.

Methods

Animals and vaccination studies. Female BALB/c mice were 7-10 weeks of age at time of vaccination. Vaccinated mice were administered the second dose 20-28 days after the initial vaccine dose. Sera and organs were collected from a cohort of mice to assess immune responses to the vaccines and the effect on bacterial burden. Mice were challenged 27-29 days post final vaccination. Mice were exposed to aerosolized (pneumonic) or SC (bubonic) challenge doses of virulent *Y. pestis* C12.

Treatment of vaccinated mice. Streptomycin was prepared in water for injection and a dose of 20 mg/kg administered to mice by the intraperitoneal (IP) route every six hours for five days. The mice were injected with the antibiotic beginning 60 h after exposure to aerosolized *Y. pestis* C12.

Results

Heterologous vaccination strategies typically protected better than live attenuated vaccines alone against a C12 challenge

Live attenuated vaccines alone are not sufficient to protect mice against plague initiated by exposure to *Y. pestis* C12 (**Figure 1**). To enhance the availability of other protective antigens but still retain the F1 mediated protection, approximately equal parts CO92 Δ*yscN* and C12 Δ*yscN* vaccine mix (Combo LAV) was selected for further evaluation in heterologous vaccine strategies to improve the protection against *Y. pestis* C12 infection. Mice which received any of the four heterologous vaccines [groups 3, 4, 7,8] were highly protected against bubonic disease when challenged four weeks after the boost vaccination, as illustrated in **Figure 2A**. Vaccination with the Combo LAV followed by rF1V + CpG, or rV +/- CpG, resulted in 100% survival [groups 4, 7, 8], $p \leq 0.001$ versus KPhos control; and 90% survival was observed in groups given either the Combo LAV first and then rF1V without CpG [group 3] or with two doses of the Combo LAV vaccine [group 2], $p = 0.0001$.

Heterologous approaches significantly improved the protection of mice exposed to aerosolized *Y. pestis* C12 (**Figure 2B**). The survival rates of these mice at both early (day 7) and final timepoints post-challenge [group 4] were significantly better (90%) than those of four of the other five groups (p values ranged from $p = 0.0001$ to $p = 0.048$). ELISA titers are listed **in Table 1**.

Tissue samples were collected from mice to assess bacterial load on day three after the aerosol challenge and results are shown in **Table 2** (n = 6/group, except as indicated). Statistical analyses revealed significant vaccine-related differences. Mice vaccinated with the heterologous Combo LAV/rF1V + CpG regimen had a GM of 274 CFU/g in the lungs and the bacterial load was significantly reduced compared to the other five groups ($p = 0.026$ to 0.0022). **Figure 3** demonstrates the utility of a LAV in contect of alayered medical countermeasure strategy that includes antibiotics.

Table 1. Total IgG antibody response against rF1V, *Y. pestis* CO92 or *Y. pestis* C12

Vaccine Group (#)[b]	n[c]	Antibody Titer[a] rF1V		TS CO92		TS C12	
Kphos (1)	4	94	(1.26)	212	(1.26)	168	(1.17)
Combo LAV x 2 (2)	5	221,106	(1.81)	52,947	(1.42)	9,263	(1.42)
Combo LAV/rF1V (3)	6	387,944	(1.47)	775,889	(1.59)	741	(2.09)
Combo LAV/rF1V + CpG (4)	6	870,906	(1.34)	29,314	(1.49)	1,008	(1.70)
KPhos/rF1V (5)	6	117,579	(1.16)	504	(1.24)	52	(1.07)
KPhos/rF1V + CpG (6)	5	265,996	(1.97)	1,676	(1.65)	120	(1.49)

[a] Values represent GM antibody titer with GSE in parentheses directed against rF1V protein, temperature shifted CO92 (TS CO92) or temperature shifted C12 (TS C12) strains.

[b] The vaccine group numbers are the same as those shown in Figures 5B - C.

[c] Number (n) of animal samples for each group. The n applies to tests with all antigens, except n=5 (instead of 6) for the Combo LAV/rF1V + CpG group tested with TS CO92 and TS C12.

Conclusions

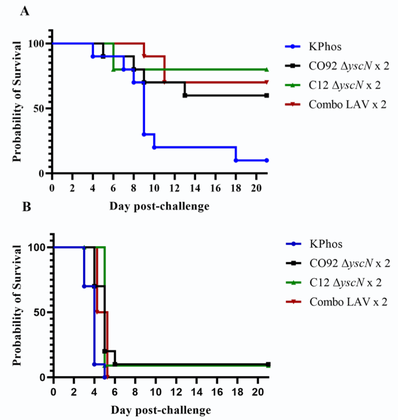

Figure 1. Survival curves of BALB/c mice vaccinated twice with the homologous LAV (days 0 and 23) and challenged four weeks later with *Y. pestis* strain C12 by the SC route (**A**) or by the aerosol route (**B**). The doses of C12 were 2.38 x 10^3 CFU (264 LD_{50}s) or 2.5 x 10^5 CFU (3.25 LD_{50}s), respectively. The mice were monitored for 21 days after challenge.

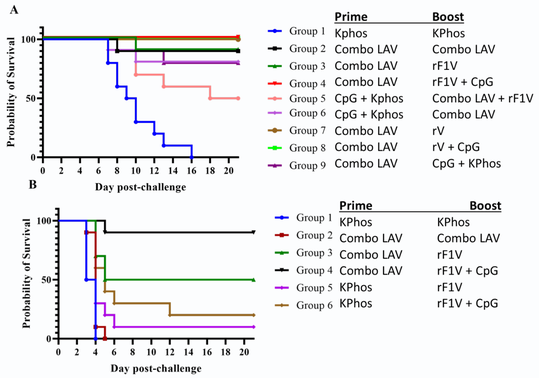

Figure 2. A comparison of protection elicited by homologous and heterologous vaccines against *Y. pestis* strain C12 challenge. The vaccine doses contained approximately 1 x 10^7 CFU total Combo LAV (ranging from 0.76 – 0.88 x 10^7 CFU) and/or 2 µg protein subunit vaccine, rF1V or rV. Two vaccinations were given 28 days apart to the mice (n = 10/group). Vaccinated mice were exposed to C12 by the SC route (3.62 x 10^3 CFU C12, 402 LD_{50}s) (**A**), or by the aerosol route with 7.93 x 10^5 CFU of C12, 10.35 LD_{50}s (**B**).

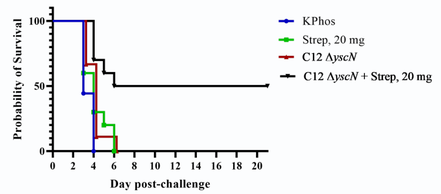

Figure 3. The protection of BALB/c mice against lethal aerosol challenge with *Y. pestis* strain C12 by a vaccination and post-challenge antibiotic treatment strategy. The mice were vaccinated as described in Figure 6 but with mutant C12 Δ*yscN* (0.48 x 10^7 and 0.70 x 10^7 CFU for doses 1 and 2 respectively); controls received KPhos alone. They were either untreated or treated with 20 mg/kg streptomycin 60h after challenge with *Y. pestis* strain C12 four weeks after the boost dose. The challenge dose was 1.67 x 10^6 CFU/mouse (21 LD_{50}s). n = 9 mice/group (KPhos alone and vaccine alone) or 10 mice/group (streptomycin alone and vaccine + streptomycin).

Table 2. The bacterial burden of mice collected three days after challenge with *Y. pestis* C12

Vaccine: Prime	Boost	Organ	# positive samples/total #[a]	CFU/g or mL[b]: GM (GSD)
KPhos	KPhos	lung	6/6	1.08 x 10^9 (2.2)
		spleen	6/6	3.98 x 10^7 (12.3)
		blood	5/5[c]	1.92 x 10^4 (5.9)
Combo LAV	Combo LAV	lung	6/6	1.43 x 10^9 (1.9)
		spleen	6/6	2.07 x 10^6 (39.3)
		blood	6/6	4.27 x 10^3 (10.0)
Combo LAV	rF1V	lung	6/6	2.36 x 10^8 (4.3)
		spleen	5/6	1.84 x 10^4 (1305.3)
		blood	3/6	8.07 x 10^2 (30.2)
Combo LAV	rF1V + CpG	lung	4/6	2.74 x 10^2 (1232.6)
		spleen	2/6	1.23 x10^2 (1577.8)
		blood	1/6	2.37 x 10^2 (19.3)
KPhos	rF1V	lung	6/6	5.29 x 10^6 (1921.7)
		spleen	4/6	4.23 x 10^3 (2960.8)
		blood	3/6	9.06 x 10^2 (20.9)
KPhos	rF1V + CpG	lung	6/6	1.16 x 10^8 (396.5)
		spleen	5/6	1.74 x 10^5 (662.4)
		blood	4/5[b]	3.21 x 10^3 (26.5)

[a]Number of samples with CFU recovered/total number mice sampled.

[b]GM and GSD values included all samples, with the LOD/SQRT(2) for those with no CFU recovered.

[c]One mouse succumbed by day 3; sample was not available.

SUMMARY

1. Heterologous approaches offer flexibility for vaccination strategies in the event of emerging or engineered threats.
2. Heterologous vaccination strategies protect mice against bubonic and pneumonic plague.
3. Live attenuated vaccines can be an important component in a layered-defense strategy.

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Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army or the Department of Defense Health Agency.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 2011. The facility where this research was conducted is fully Accredited by the Association for Assessment and Accreditation Of Laboratory Animal Care International.

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