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Yersinia pestis CO92Δ*yopH* Is a Potent Live, Attenuated Plague Vaccine▽

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An in-frame deletion of the *yopH* gene in *Yersinia pestis* CO92 attenuates virulence in both bubonic and pneumonic plague models. When it is used as a live, attenuated vaccine, CO92Δ*yopH* provides a high degree of protection from parental and respiratory challenge with *Y. pestis* CO92.

Yersinia pestis is the causative agent of plague, a potentially contagious disease that generally manifests as either the bubonic or pneumonic form of the disease, depending on the route of infection. The historical abilities of *Y. pestis* to cause epidemic and pandemic disease are well documented, and in recent years, there have been small naturally occurring outbreaks of both bubonic and primary pneumonic plague (4, 5, 30). Although *Y. pestis* remains susceptible to antibiotics, the identification of naturally occurring multiple-drug-resistant strains of *Y. pestis* in Madagascar (13, 15) and the discovery that the high-frequency conjugative transfer of plasmids containing drug resistance from coinhabiting bacteria to *Y. pestis* occurs in the flea midgut (18) highlight the importance of finding an effective plague vaccine.

Humankind has been using killed whole-cell vaccines against *Y. pestis* since the 1890s, with the USP, formalin-killed plague vaccine being in use in the United States until 1999 (36). This vaccine provides protection against bubonic plague, but there is good evidence that the vaccine provides little protection against primary pneumonic plague (9, 27, 37), and adverse side effects are known to occur (25, 32). A live, attenuated vaccine with particular focus on the use of the EV76 pigmentation-negative *Y. pestis* strain has been developed; however, severe side effects have been observed (27, 33), as have various levels of virulence among host species (8, 16, 28).

Currently, protein subunit vaccines which include both the type III secretion system protein LcrV and the capsular antigen F1 are under development. The subunit vaccine provides excellent protection against bubonic and pneumonic plague in animal models and is well tolerated by humans (1, 14, 17, 19–21, 38). While the development of the subunit vaccine has been very successful, the limited antigenic complexity of the vaccine, coupled with the diversity of *Y. pestis* strains (3), suggests that an improved live, attenuated vaccine may provide more universal protection or could be coupled with the subunit vaccine to provide greater protection against *Y. pestis*.

YopH is a protein tyrosisne phosphatase that is a type III secretion system effector protein encoded on the pCD1 virulence plasmid of *Y. pestis* (10, 29). Previous studies have determined that YopH is an essential virulence factor in murine models of enteropathogenic infections with yersiniae (6, 24), as well as systemic models of *Y. pestis* (KIM5, *pgm* negative) infection (22). These studies demonstrated that infection with a *yopH* mutant resulted in an avirulent or highly attenuated phenotype. It is notable that these studies also showed that *yopH* mutants were able to colonize intestinal tissues, although there was a defect in the ability of *yopH* mutants to colonize/disseminate to the spleens and livers (24, 34) or to the mesenteric lymph nodes (24) of infected mice. Altogether these data suggest that *yopH* mutants would be a reasonable live, attenuated *Y. pestis* vaccine strain.

An in-frame *yopH* deletion mutation in *Y. pestis* CO92 was created. For our studies, 6- to 8-week-old female outbred CD1 mice (Charles River Laboratories, Wilmington, MA), a strain

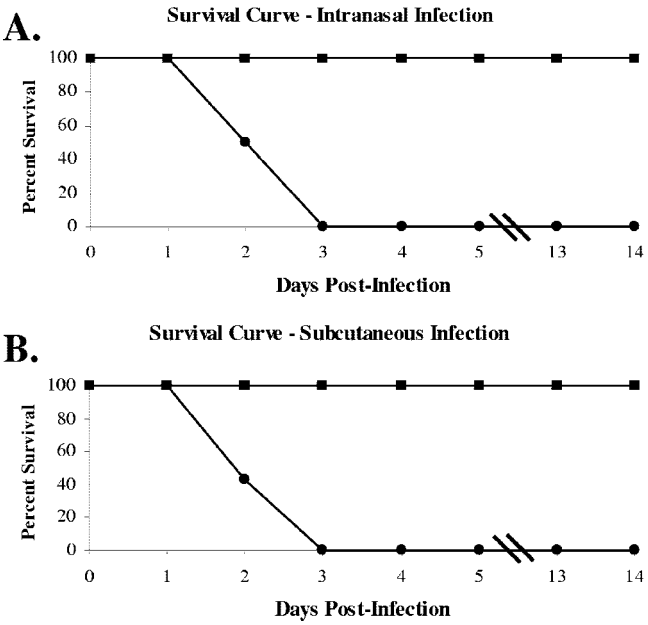

FIG. 1. Survival curve. Six- to 8-week-old female CD1 mice were infected as follows: i.n. with *Y. pestis* CO92 (~10^5 CFU [●]) or i.n. with *Y. pestis* CO92Δ*yopH* (~10^7 CFU [■]) (A) or s.c. with *Y. pestis* CO92 (~10^5 CFU [●]) or s.c. with *Y. pestis* CO92Δ*yopH* (~10^7 CFU [■]) (B). The mice were then monitored for survival every 12 h for 14 days. These data represent the results of three independent experiments obtained with a total of 25 mice (A) and 20 mice (B) per group.

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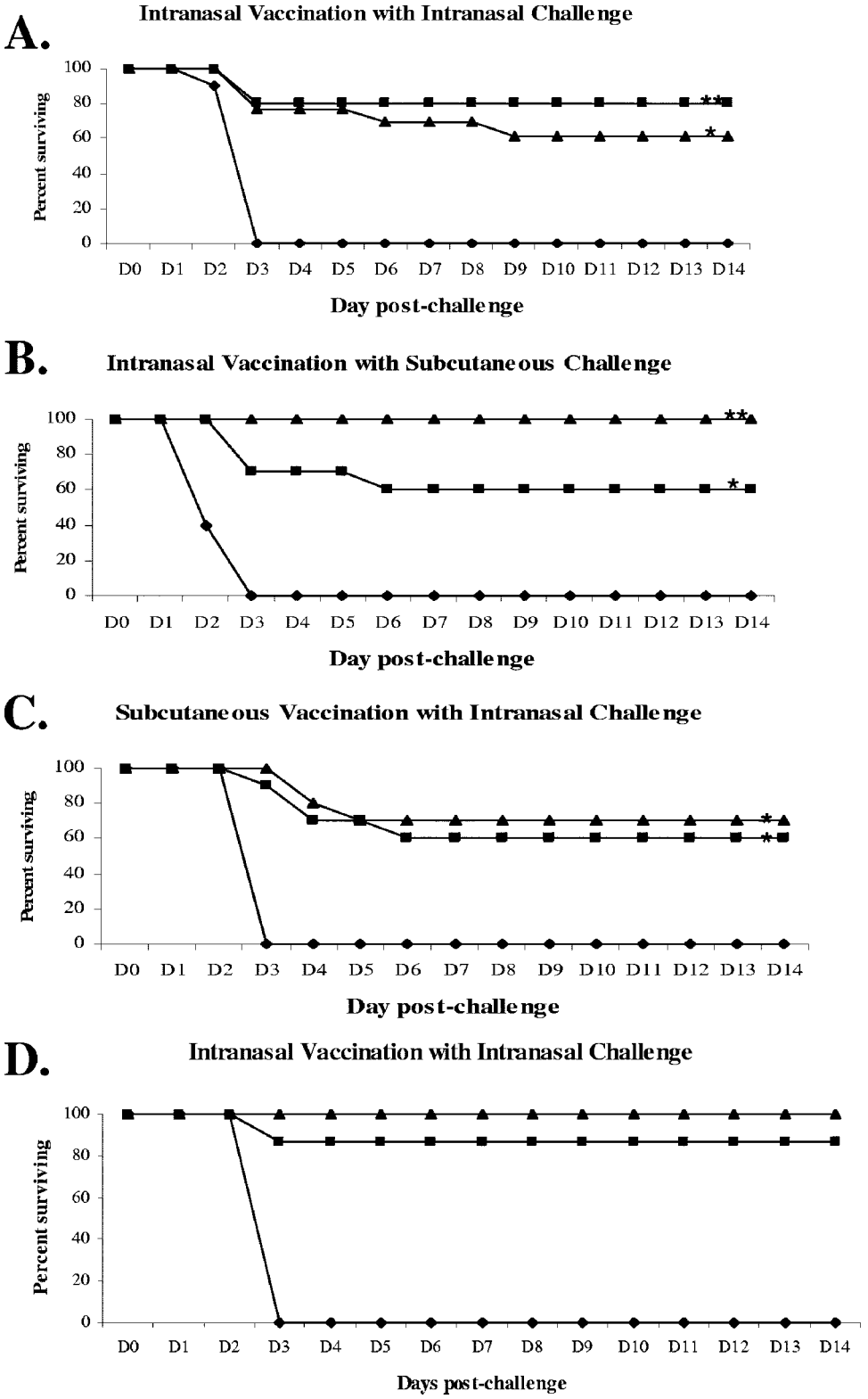

FIG. 2. Vaccination and challenge. Six- to 8-week-old female CD1 mice were vaccinated with *Y. pestis* CO92Δ*yopH* (A to C) or the *Y. pestis* CO92 *pgm* mutant (D) i.n. with ~10^5 CFU (■), i.n. with ~10^7 CFU (▲), s.c. with ~10^5 CFU (■), or s.c. with ~10^7 CFU (▲) or were mock vaccinated either i.n. or s.c. with saline (◆). After 22 days, the mice were challenged with ~10^5 CFU CO92 by either an i.n. or an s.c. route. These data represent the results of two independent experiments with a total of 13 to 15 mice (A), 10 mice (B and C), and 15 mice (D) per group.

commonly used in vaccine studies, were infected with CO92 or CO92Δ*yopH* intranasally (i.n.) or subcutaneously (s.c.), as we have described recently (7). As a control, an avirulent *Y. pestis* CO92 pigmentation-negative mutant (*pgm* mutant) was used for i.n. vaccinations with a subsequent i.n. challenge with *Y. pestis* CO92. The CO92 pigmentation-negative strain is expected to give results similar to those obtained with other live, attenuated vaccines based on this mutation, such as EV76 (33). All experiments were performed at biosafety level 3, in accordance with the Institutional Biosafety Committee- and the Institutional Animal Care and Use Committee-approved protocols. *Yersinia pestis* CO92 was obtained from the Centers for Disease Control and Prevention Select Agent Distribution Activity (Fort Collins, CO).

In both a primary pneumonic plague model and a bubonic plague model of *Y. pestis* infection, 100% of mice infected i.n. or s.c. with $\sim 10^5$ CFU (data not shown) or $\sim 10^7$ CFU CO92Δ*yopH* (Fig. 1) survived. These challenge doses correspond to 833 to 83,333 CO92 50%-lethal-dose (LD_{50}) equivalents in an i.n. route of infection, respectively (7, 23), suggesting that CO92Δ*yopH* is severely attenuated. All of the animals infected with the parental CO92 strain ($\sim 10^5$ CFU) died within 3 days (Fig. 1). Mice infected with CO92Δ*yopH* by the i.n. route with either dose showed no overt signs of illness (data not shown). Mice infected with CO92Δ*yopH* by the s.c. route showed no overt signs of illness and showed no reaction at the injection site when they were infected with $\sim 10^5$ CFU; however, when they were infected with $\sim 10^7$ CFU, the mice exhibited signs of physical illness, including ruffled fur and inactivity, as well as an injection site reaction, resulting in limited use of the leg where the injection occurred. This was a transient side effect, however, that lasted approximately 10 days (data not shown). Mice infected i.n. with $\sim 10^5$ CFU of *pgm*-negative strain CO92 showed no signs of illness, while those infected i.n. with $\sim 10^7$ CFU appeared lethargic and inactive and had ruffled fur for approximately 10 days after infection (data not shown). Consistent with the observations made with the *yopH* mutants of the enteropathogenic yersiniae (11, 19), CO92Δ*yopH* could be cultured from the site of infection (from the lungs following an i.n. infection [data not shown]) but failed to disseminate to the spleen and liver (data not shown). Altogether these data suggest that even at high challenge doses CO92Δ*yopH* is severely attenuated and is unable to cause plague-like disease.

In addition, these data would suggest that CO92Δ*yopH* could serve as a live, attenuated vaccine strain. The ideal vaccine against *Y. pestis* would protect an individual from infection with *Y. pestis* via multiple routes of inoculation and with minimal side effects after a single immunization. With this in mind, using CO92Δ*yopH* as a live, attenuated vaccine, we investigated the different combinations of i.n. or s.c. vaccination using CO92Δ*yopH* with either the i.n. or the s.c. challenge route. Mice were vaccinated by either an i.n. or an s.c. route with a $\sim 10^5$ CFU or a $\sim 10^7$ CFU dose of CO92Δ*yopH*. As a comparison, mice were vaccinated i.n. with $\sim 10^5$ or $\sim 10^7$ CFU of *pgm*-negative strain *Y. pestis* CO92. At 22 days after vaccination, the mice were challenged by either an i.n. or an s.c. route with $\sim 10^5$ *Y. pestis* CO92. Postchallenge survival was monitored for 14 days. Statistical analysis was performed by Fisher's exact test. For the control vaccine, a single i.n. vaccination with $\sim 10^5$ CFU of the *Y. pestis* CO92 *pgm* mutant provided 86.6% protection and a single i.n. vaccination with $\sim 10^7$ CFU provided 100% protection against i.n. challenge with $\sim 10^5$ CFU CO92. A single i.n. vaccination with $\sim 10^5$ CFU CO92Δ*yopH* provided approximately 80% protection against i.n. challenge ($P = 0.0001$) and approximately 60% protection against s.c. challenge ($P = 0.0108$) with $\sim 10^5$ CFU of CO92 (Fig. 2). Likewise, s.c. vaccination with $\sim 10^5$ CFU CO92Δ*yopH* provided approximately 60% protection against i.n. challenge ($P = 0.0108$) with $\sim 10^5$ CFU CO92 (Fig. 2C). s.c. vaccination with $\sim 10^7$ CFU CO92Δ*yopH* improved the rate of survival to 70% when the mice were challenged with a high dose ($\sim 10^5$ CFU) of wild-type *Yersinia pestis* CO92 ($P = 0.0031$). Interestingly, mice vaccinated i.n. with $\sim 10^7$ CFU of CO92Δ*yopH* were 100% protected against subsequent s.c. challenge ($P < 0.0001$) and were 61.5% protected against subsequent i.n. challenge ($P = 0.0016$) with $\sim 10^5$ CFU CO92. These data suggest that a single mucosal vaccination with CO92Δ*yopH* can provide systemic protection against a high-dose ($\sim 10^5$-CFU) virulent *Y. pestis* challenge. CO92Δ*yopH* is capable of providing protection via two routes of vaccination and against two routes of infection (Fig. 2A to D).

This study suggests CO92Δ*yopH* is a suitable live, attenuated vaccine strain that protects against high-dose challenge with fully virulent *Y. pestis* strain CO92 by either parenteral or aerosol challenge after a single immunization. Both the parenteral and the mucosal routes of immunization provided significant protection, but the mucosal route of immunization provided the highest degree of protection against both a mucosal and a parenteral challenge. The control vaccine, which comprised *pgm*-negative strain CO92, also provided a high level of protection. Both strain CO92Δ*yopH* and *pgm*-negative strain CO92 offered similar levels of protection against i.n. challenge with doses as high as 830 LD_{50}s. These data suggest that the CO92Δ*yopH* strain may be as good a live, attenuated plague vaccine as the benchmark EV76 strain.

Live, attenuated vaccines always raise concerns about safety, as illustrated by the virulence of EV76 in nonhuman primates (8, 28). However, previous live, attenuated plague vaccines had unsure lineages and the attenuating mutations are often uncertain (11, 35). CO92Δ*yopH* has a well-defined lineage, DNA sequence, and attenuating mutation (12, 22, 31). Consistent with the previous observations made with the enteropathogenic yersiniae (6, 24, 34), the *Y. pestis yopH* mutation is sufficient to severely attenuate CO92 while maintaining its ability to induce protective immune responses in mice. The potential safety and efficacy of this strain as a live, attenuated vaccine could be enhanced further with additional defined attenuating mutations.

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