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Recombinant V Antigen Protects Mice against Pneumonic and Bubonic Plague Caused by F1-Capsule-Positive and -Negative Strains of *Yersinia pestis*

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The purified recombinant V antigen from *Yersinia pestis*, expressed in *Escherichia coli* and adsorbed to aluminum hydroxide, an adjuvant approved for human use, was used to immunize outbred Hsd:ND4 mice subcutaneously. Immunization protected mice from lethal bubonic and pneumonic plague caused by CO92, a wild-type F1$^+$ strain, or by the isogenic F1$^-$ strain C12. This work demonstrates that a subunit plague vaccine formulated for human use provides significant protection against bubonic plague caused by an F1$^-$ strain (C12) or against substantial aerosol challenges from either F1$^+$ (CO92) or F1$^-$ (C12) *Y. pestis*.

Yersinia pestis is the etiological agent of bubonic and pneumonic plague. The current plague vaccine USP, which is licensed for human use in the United States and the United Kingdom, relies on the *Y. pestis*-specific F1 capsule protein as the principal immunogen (26). While indirect evidence suggests that the vaccine was effective in preventing bubonic plague in U.S. soldiers in Vietnam, pneumonic cases did occur (10, 24). This Formalin-killed, whole-cell vaccine offers little protection to guinea pigs and mice (4, 31, 36) or monkeys (30) challenged by aerosol with wild-type F1$^+$ *Y. pestis* and essentially no protection to mice challenged with an F1$^-$ *Y. pestis* strain (18, 47). A Formalin-killed, whole-cell preparation similar to the plague vaccine USP protected nonhuman primates challenged intratracheally from pneumonic plague (16). This preparation used the Yreka instead of the 195/P strain of *Y. pestis* which is used in the plague vaccine USP. Whether protection from pneumonic plague was due to the method of challenge or differences in antigen expression of the two strains is unknown, but the data suggest that partial protection against pneumonic plague by wild-type *Y. pestis* whole-cell vaccine was possible.

A live, attenuated *Y. pestis* vaccine strain (EV76) has also been used as a human vaccine and was recently compared with the plague vaccine USP in inbred mice. While the live EV76 vaccine conferred greater protection against infection with wild-type *Y. pestis*, this vaccine caused severe adverse reactions in the mice (32). Variable virulence of the live vaccine strains in animal models and reactogenicity in humans has prevented this vaccine from gaining worldwide acceptance (24, 42).

Recently, isogenic F1$^-$ strains of *Y. pestis* were derived from fully virulent wild-type strains (14, 15, 48). These F1$^+$-F1$^-$ isogenic strain pairs were shown collectively to be virulent for mice (14, 48), guinea pigs (14), and African green monkeys (12). These genetically characterized variants confirmed earlier studies in which phenotypically defined F1$^-$ strains were isolated (8, 43). These findings identified the need for a plague vaccine that could protect against F1$^-$ variants, even though only one phenotypically F1$^-$ organism has ever been reported from a fatal human case (46). Further, passive immunization with F1 monoclonal antibodies (2) or active immunization with the recombinant F1 protein (4) protected mice against fatal disease; however, F1$^-$ variants were isolated from the spleens of some survivors. A vaccine composed of multiple immunogens should reduce the possibility of selecting variants that could produce a chronic infection and should protect against F1$^-$ strains.

Burrows and Bacon originally suggested that the V antigen of *Y. pestis* could be an important immunogen (9). Later, Lawton et al. (21) demonstrated that polyclonal antiserum containing V antibody or active immunization with partially purified V antigen, could protect against a virulent *Y. pestis* parenteral challenge. More recently, protection obtained by using polyclonal monospecific V sera (40) or V monoclonal antibodies (27, 35) confirmed the ability of V antibody to passively protect against a *Y. pestis* challenge.

Brubaker et al. (7) reported the V antigen to be unstable. Therefore, they developed a protein A-V fusion protein to stabilize the V protein and showed that immunization with this fusion protein was efficacious against an attenuated *Y. pestis* strain that was lethal to mice inoculated intravenously (28). More recently, Leary et al. (22) demonstrated that a recombinant V protein (rV) is not autoproteolytic and, when combined with incomplete Freund's adjuvant, protected mice against a subcutaneous (s.c.) challenge with 10^6 CFU of a virulent wild-type *Y. pestis* strain. These studies suggested that the use of rV in an improved subunit plague vaccine was feasible against bubonic plague from wild-type *Y. pestis*.

In this study, we extended the previous finding that rV protects against bubonic plague by demonstrating that, when combined with an approved-human-use adjuvant to replace incomplete Freund's adjuvant, rV could protect against an F1$^-$ *Y. pestis* challenge and also protect against pneumonic plague caused by either an F1$^-$ or a wild-type F1$^+$ strain of *Y. pestis*.

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MATERIALS AND METHODS

Bacterial strains and cultivation. The *Y. pestis* wild-type CO92, biovar orientalis, isolated directly from the sputum of a human case of pneumonic plague (13) was provided by T. Quan, Centers for Disease Control and Prevention, Ft. Collins, Colo. C12 is an F1$^-$ isogenic strain derived by site-directed mutagenesis of the F1 structural gene from the CO92 strain (48). During the selection process, the C12 strain was twice passaged in mice. Stocks of the two strains were frozen at −70°C in 66% glycerol in identical single-use aliquots. For challenge studies, frozen stocks were streaked onto tryptose blood agar base (Difco Laboratories, Detroit, Mich.) slants and incubated at 28°C for 2 days. Bacterial cells were harvested from the slants in 5 ml of heart infusion broth (HIB; Difco) and adjusted to an A_{620} of 1.0 (approximately 10^9 CFU/ml). For s.c. challenges, the suspension was diluted in HIB to various multiples of the 50% lethal doses (LD_{50}) for CO92 (61-, 6,100-, or 1,200,000 ×) and C12 (37-, 3,700-, or 740,000 ×), respectively, and 0.2 ml was injected. For aerosol challenges, 2 ml of the adjusted slant suspension was used to inoculate flasks containing 100 ml of HIB supplemented with 0.2% xylose. The broth cultures were grown for 24 h in a 30°C shaker at 100 rpm, centrifuged, washed twice with HIB, adjusted to an A_{620} of 10.0 (approximately 10^{10} CFU/ml), and diluted to various multiples of the LD_{50} for CO92 (59- or 971×) and for C12 (84- or 193×). Antifoam A emulsion (Sigma Chemical Company, St. Louis, Mo.) was added to a final concentration of 0.2% (vol/vol) in the bacterial suspension just before the aerosol challenges.

rV preparation. The expression and purification of rV was previously described (22). Briefly, the *IcrV* gene encoding the V antigen was amplified from *Y. pestis* GB DNA by PCR. The amplified product was cloned into the pGEX-5X-2 plasmid downstream of the glutathione *S*-transferase (GST) gene and electroporated into *Escherichia coli* JM109 cells. The recombinant plasmid pVG100 in *E. coli* JM109 cells produced a GST-V fusion protein. The cells were lysed and centrifuged, and the supernatant was mixed with glutathione Sepharose 4B. This material was packed into a column, and the GST-V fusion protein was eluted with a Tris buffer containing glutathione. The fusion protein was cleaved with factor Xa. Cleaved GST and uncleaved GST-V were separated from rV by affinity adsorption. The estimated molecular weight of purified rV was 36,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Animal immunizations. Female 8- to 9-week-old outbred Swiss Webster mice (Hsd:ND4) were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). Experiments with animals were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (29). Mice were given food and water ad libitum. Mice were immunized s.c. with 10 μg of rV adsorbed to an aluminum hydroxide adjuvant (1.3% Alhydrogel, lot no. 4023; Superfos Biosector, Vedbaek, Denmark) on day 0 and again on day 30. The rV in Dulbecco's phosphate-buffered saline (PBS) was gently mixed overnight at 4°C with the aluminum hydroxide adjuvant and checked for complete adsorption by assaying for residual protein (BCA; Pierce, Rockford, Ill.) after centrifugation at 2,000 × *g* for 5 min. The concentration of rV was adjusted with PBS to yield a final concentration of 10 μg of rV per 0.19 mg of aluminum per 0.2-ml dose.

Mice were individually identified by transponders with unique numbers (Bio-Medic Data Systems, Inc., Maywood, N.J.). The transponders were injected s.c. near the scruff of the neck 1 week before immunization so that the responses of individual mice to immunization and challenge could be determined. This positive identification transponder is essentially inert when injected s.c. (5).

Measurement of serum antibody titers. Blood was obtained from each mouse on day 51 postimmunization, 7 days before *Y. pestis* challenge. Mice were anesthetized with a mixture of 5 mg of xylazine (XYLA-JECT; Phoenix Pharmaceutical, Inc., St. Joseph, Mo.) per kg, 0.83 mg of acetylpromazine (Fermenta Animal Health Co., Kansas City, Mo.) per kg, and 67 mg of ketamine hydrochloride (Ketamine; Phoenix Pharmaceutical, Inc.) per kg administered intramuscularly. Blood was obtained from the retro-orbital sinus. Blood was also collected by cardiac puncture on day 28 postchallenge from the mice that survived a lethal infection. A separate group of mice was immunized as above but were bled on days 13, 29, and 58 postimmunization to measure the serum antibody titers and to determine the V-specific immunoglobulin G (IgG) antibodies in lung lavage fluid on day 58. Lavage fluid was collected after euthanizing the mice by exposing the trachea, inserting and securing an 18-gauge needle, and aspirating 1 ml of PBS three times before final withdrawal.

Total IgG, IgG1, IgG2a, and IgG2b serum antibody titers to the V antigen were determined by using a modification of an enzyme-linked immunosorbent assay (ELISA) (39). Briefly, 0.1 μg per well of purified rV in ammonium bicarbonate buffer, pH 8.8, was added to 96-well plates (Linbro; ICN Flow, Horsham, Pa.) and air dried. Plates were blocked with PBS containing 0.1% bovine serum albumin (Sigma) and 0.05% gelatin (Difco). These plates were washed three times in PBS with 0.3% Tween 20, and twofold serial dilutions of the samples from 1:640 to 1:1,310,720 were applied in triplicate. After 2 h at 37°C, the plates were washed and a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry, Gaithersburg, Md.) was applied for 1 h at 37°C. Plates were washed and the chromogenic substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) was added. The reaction was stopped after 30 min with 10% SDS, and the plates were read at an optical density at 405 nm (OD_{405}). To determine IgG subclass, goat anti-mouse IgG1, IgG2a, or IgG2b (Sigma) was allowed to react with the bound test sera for 1 h and a peroxidase-conjugated rabbit anti-goat IgG (Sigma) was used to detect the isotypes. Each of these antibodies was used at a maximum dilution of 1:3,000. The endpoint titer was defined as the highest test serum dilution giving a reading of ≥0.1 OD units after subtracting the background from the wells without antigen. The results obtained are expressed as the geometric mean titer (GMT) or $\log_{10}$ mean ± standard deviation (reciprocal endpoint titers). In the case of individual titers of <640, i.e., the initial dilution, the convention of using half of this value for statistical purposes was used.

Challenge with *Y. pestis*. Groups of 10 immunized and 10 control mice were challenged s.c. with 0.2-ml aliquots of the CO92 or the C12 strain of *Y. pestis* at various cell concentrations as noted in Results. The s.c. LD_{50} for adult mice are 1.9 and 9.1 CFU, calculated at day 14 postinfection, for CO92 and C12, respectively (41, 48).

Mice were exposed to a small-particle aerosol (mass median aerosol diameter of 1.2 μm) in a modified Henderson exposure system (19) contained within a Class III biological safety cabinet. The aerosol was generated with a Collision nebulizer (23) driven by compressed air at 26 lb/in^2 with a flow rate of 7.5 liter/min. Up to 25 unanesthetized mice were challenged at a time in the nose-only aerosol exposure system. Mice from the various groups were divided between different aerosol exposures to minimize any exposure differences. Mice were exposed to the infectious aerosol for 10 min. The aerosol LD_{50} of the CO92 and C12 strains for adult mice were 2×10^4 (41) and 1.1×10^5 CFU, calculated at day 14, respectively. A sample of the aerosolized *Y. pestis* was collected in 10 ml of HIB with an all-glass impinger during each exposure. The challenge doses were assayed by plating dilutions of the collection fluid from the all-glass impingers on sheep blood agar. The inhaled doses were estimated by using Guyton's formula (17).

The mice were observed daily for 28 days, at which time the survivors were anesthetized and exsanguinated by cardiac puncture, and the spleens were removed aseptically, weighed, and titrated for viable *Y. pestis* cells. Ten percent (wt/vol) spleen homogenates were prepared with Ten Broeck homogenizers and plated onto sheep blood agar plates.

SDS-PAGE and Western blot (immunoblot) analysis. To determine the synthesis of the V antigen, *Y. pestis* colonies isolated on sheep blood agar plates from the spleens of mice that survived the 28-day observation period were inoculated into 5 ml of chemically defined thiosulfate-modified Higuchi medium (38) and incubated overnight at 26°C at 150 rpm. The cells were subcultured into fresh thiosulfate-modified Higuchi medium without Ca^{+2} to an OD_{600} of 0.2 with shaking for 2 h at 26°C and then shifted to 37°C for 6 h to induce the expression of V antigen. The cultures were centrifuged at 5,000 × *g* for 5 min, and the pellets were resuspended in PBS. The pellet suspension and the supernatant were frozen at −20°C until assayed. Samples were mixed with an equal volume of 2× SDS-PAGE loading buffer and heated at 100°C for 10 min. Proteins were separated on 10 to 20% Tricine gels (Novex, San Diego, Calif.) and transferred to nitrocellulose membranes. The V protein was detected by Western blot with a mouse polygonal monospecific antibody (provided by G. Andrews, USAMRIID, Ft. Detrick, Frederick, Md.) to rV and was detected by the ECL system (Amersham, Arlington Heights, Ill.)

DNA preparation. Overnight cultures of *Y. pestis* strains were prepared by inoculating growth from a sheep blood agar plate into 3 ml of HIB supplemented with 0.2% xylose. Plasmid DNA samples were prepared from these cultures by a modification of the method described by Kado and Liu (33). After removing residual phenol in Insta Mini Prep tubes (5 Prime-3 Prime, Inc., Boulder, Colo.), the aqueous layer was gently extracted with chloroform in the same tube. The aqueous layer was ethanol precipitated and resuspended in 50 μl of 10 mM TRIS, pH 8.0, with 1 mM EDTA.

PCR. The following primer pair specific for the *hms* locus of *Y. pestis* was used to confirm the identification of the cells: 5′CCGCCCTGTGCTGTCTTC3′ (95 to 22) and 5′CAACACAACCCACGGGG3′ (95 to 23). Primer sequences were designed by using the *hms* sequence submitted to GenBank by Lillard et al. (GenBank accession number U22837). The reactions contained the following components: 1 to 2 μg of total DNA, all four deoxynucleotide triphosphates at 200 μM each, 1× *Taq* polymerase buffer, 2 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (all reagents were from GIBCO BRL Life Technologies, Gaithersburg, Md.). Samples were denatured for 1 min at 94°C before the *Taq* polymerase was added. Samples were overlayed with a drop of mineral oil. After a 5-min 94°C denaturation cycle, samples were subjected to 30 amplification cycles. The final cycle ended with a 10-min extension period at 70°C. Amplification cycles consisted of a 1-min denaturation at 94°C, a 2-min annealing at 65°C, and a 3-min extension period at 70°C.

Statistics. Student's *t* test, analysis of variance, and least significant difference tests were used to compare the various treatment groups at the 0.05 level of probability (34).

RESULTS

Protection against an s.c. challenge with *Y. pestis*. An earlier study demonstrated the efficacy of rV combined with incomplete Freund's adjuvant against a parenteral challenge (22). We determined the protective efficacy of rV adsorbed to an

TABLE 1. Survival of Hsd:ND4 mice immunized with rV and challenged with *Y. pestis*[a]

Challenge dose[b]	Survival ratio[c]	MTD ± SD (days)[d]
rV + Alhydrogel, CO92, s.c.		
61	10/10	
6,100	10/10	
1,200,000	8/10	5.0 ± 1.4
Alhydrogel alone, CO92, s.c.		
61	1/10	6.1 ± 0.9
6,100	0/10	4.8 ± 1.3
rV + Alhydrogel, C12, s.c.		
37	10/10	
3,700	10/10	
740,000	9/10	7.0 ± 0.0
Alhydrogel alone, C12, s.c.		
37	1/10	11.4 ± 7.0
3,700	0/10	7.2 ± 5.5
740,000	0/10	5.3 ± 1.0
rV + Alhydrogel, CO92, aerosol		
59	9/10	4.0 ± 0.0
971	10/10	
Alhydrogel alone, CO92, aerosol		
59	0/9	3.3 ± 0.5
971	0/10	3.0 ± 0.0
rV + Alhydrogel, C12, aerosol		
84	7/8	11.0 ± 0.0
193	9/10	5.0 ± 0.0
Alhydrogel alone, C12, aerosol		
84	0/9	3.7 ± 0.7
193	0/10	3.1 ± 0.3

[a] Female Swiss Webster (Hsd:ND4) mice were immunized s.c. with 10 μg of rV adsorbed to aluminum hydroxide adjuvant (Alhydrogel) or adjuvant alone on days 0 and 30. The mice were challenged on day 58 postimmunization.
[b] Challenge doses are multiples of the LD_{50} of the CO92 or C12 strains as indicated. Treatment groups and challenge route are also indicated.
[c] Survived/total inoculated at day 28 postinfection. Ratio of survivors to total number of inoculated mice was determined at day 28 postinfection.
[d] Mean time-to-death ± standard deviation.

adjuvant approved for human use, aluminum hydroxide, against the wild-type $F1^+$ CO92 strain of *Y. pestis*. Groups of 10 rV-immunized or control mice were challenged s.c. with various doses of wild-type, $F1^+$ CO92, ranging from 61 to 1.2×10^6 times the LD_{50} (61 to 1.2×10^6 LD_{50}) 4 weeks after the second immunization (Table 1). All rV-immunized mice survived challenge with doses of 61 or 6.1×10^3 LD_{50}; 80% of the mice survived a challenge of 1.2×10^6 LD_{50}. All control mice, except one in the 61 LD_{50} group, died. This mouse seroconverted to rV with a titer of 2,560.

Because immunization with rV was efficacious against wild-type *Y. pestis*, we examined the protection afforded by rV against $F1^-$ C12, which is incapable of producing the F1 capsular protein. Mice were challenged with doses ranging from 37 to 7.4×10^5 LD_{50} of the nonencapsulated C12 strain. All rV-immunized mice survived the parenteral challenge of 37 or 3.7×10^3 LD_{50}, and 90% of the mice survived when challenged with 7.4×10^5 LD_{50}. All C12-challenged control mice died except one mouse in the 37 LD_{50} group. The surviving control mouse had a postchallenge anti-rV titer of 1:163,840, indicating that it survived infection. These results demonstrated that immunization with rV adsorbed to aluminum hydroxide could protect mice against needle-inoculated plague caused by both $F1^+$ and $F1^-$ strains of *Y. pestis*.

The spleens from 55 rV-immunized mice which survived an s.c. challenge with either CO92 or C12 were examined for the presence of viable *Y. pestis* cells at 28 days postinfection. Ten percent (wt/vol) spleen homogenates were plated on sheep blood agar and examined after 2 days of incubation at 28°C. Only 1 of the 55 homogenates examined was positive for viable organisms. This mouse had been infected with 3.7×10^3 LD_{50} of the C12 strain. Its spleen had a bacillary burden of 1.6×10^3 CFU/g of tissue. The one Alhydrogel control mouse that survived a challenge dose of 37 LD_{50} also contained viable *Y. pestis* on day 28 postinfection. The identity of these isolates as *Y. pestis* was confirmed by PCR. These isolates were still capable of producing the V protein, as determined by Western blot with a monospecific V antiserum.

While almost all mice cleared their infections, increases in spleen weight suggested that most of the CO92-challenged groups had survived an active bacterial infection. The mean spleen weight of the control mice ($n = 10$) was 0.08 ± 0.01 g, while spleen weights of the ascending doses of CO92-infected mice were 0.20 ± 0.06 g ($P = 0.0066$), 0.22 ± 0.07 g ($P = 0.0008$), and 0.11 ± 0.02 g ($P = 0.5335$), respectively. However, no significant differences ($P \leq 0.05$) compared with control mice were detected for the spleen weights of the C12-infected mice, which were 0.12 ± 0.03 g, 0.16 ± 0.03 g, and 0.12 ± 0.03 g in ascending dose order, respectively.

Protection against an aerosol challenge with *Y. pestis*. Having demonstrated the efficacy of rV to protect against needle-inoculated plague, we challenged mice by aerosol with the wild-type, $F1^+$ CO92 or the $F1^-$ C12 strain to determine whether immunization with rV was efficacious against pneumonic plague (Table 1). Groups of mice were challenged in the nose-only aerosol apparatus with either 59 or 971 LD_{50} of the CO92 strain. Immunization afforded 90% (9 of 10) protection against challenge with 59 LD_{50} and 100% (10 of 10) protection against 971 LD_{50}. The increased survival in the high-dose group is not statistically different from the low-dose group and most likely reflects the small number of animals in each challenge group. Mice were similarly protected when challenged with the $F1^-$ strain, C12, with 88% (7 of 8) surviving a challenge dose of 84 LD_{50} and 90% (9 of 10) surviving when challenged with 193 LD_{50}. *Y. pestis* could not be isolated from the spleens of the 35 mice immunized with rV which survived either a CO92 or C12 aerosol challenge. All control mice died.

Spleen weights of the ascending challenge dose groups for the CO92-infected mice were 0.15 ± 0.05 g ($P = 0.1182$) and 0.19 ± 0.06 g ($P = 0.0076$), respectively. The spleen weights of the ascending challenge dose groups for the C12-infected mice were 0.21 ± 0.08 g ($P = 0.0039$) and 0.23 ± 0.2 g ($P = 0.0009$), respectively. Immunization with rV was efficacious against pneumonic plague, which has a more rapid disease progression than bubonic plague in mice.

Ontogeny and correlation of protection with V antibody. A group of 10 mice, separate from the mice that were challenged, were immunized on days 0 and 30 with 10 μg of rV adsorbed to aluminum hydroxide and assayed for their V-ELISA titers. The mice were bled on days 13, 29, and 58 postimmunization, and the IgG, IgG1, IgG2a, and IgG2b V-ELISA titers were determined on serum from individual mice. Additionally, lung lavage fluid was collected from each mouse on day 58 postimmunization to determine whether IgG anti-V antibody was detectable in the lung. By day 13 postimmunization, 100% ($n = 10$) of the mice developed detectable IgG V-ELISA titers with a GMT of 8,317 (Table 2). The titer increased to 52,780 by day 29 and was 640,000 on day 58 postimmunization, the day of challenge for the other groups. The titer of the control group was <640, the initial serum dilution tested. The IgG anti-V GMT of the lung lavage fluid was 788 for the immunized mice and <5 for the controls. The initial dilution tested for the lavage fluid was 1:5. IgG anti-V antibodies were detectable in

TABLE 2. Ontogeny of IgG ELISA titers of mice immunized with rV[a]

Day postimmunization	IgG ELISA titer			
	IgG	IgG1	IgG2a	IgG2b
13	3.92 ± 0.15	3.23 ± 0.43	2.66 ± 0.29	2.66 ± 0.29
29	4.72 ± 0.25	4.52 ± 0.29	3.02 ± 0.40	3.26 ± 0.50
58	5.81 ± 0.20	5.79 ± 0.30	4.34 ± 0.17	4.34 ± 0.36

[a] Ten female Swiss Webster (Hsd:ND4) mice were immunized s.c. with 10 μg of rV adsorbed to an aluminum hydroxide adjuvant (Alhydrogel) on days 0 and 30. Mice were bled on days 13, 29, and 58 postimmunization. Log_{10} reciprocal endpoint titers were determined for individual mice and the mean ± standard deviation is presented for each group of animals. No specific antibodies were detected in the sera of control mice.

all of the lung lavage fluids from rV-immunized mice. The IgG subclass V-ELISA responses are shown in Table 2. The IgG1 V-ELISA titer was similar to total IgG and predominated over IgG2a or IgG2b.

The IgG rV ELISA antibody titers of 40 rV-immunized mice were examined at day 51 postimmunization. All 40 of the mice, including the mice that died, responded to two doses of the vaccine. The reciprocal rV titers ranged from 81,920 to 1,310,720. Total IgG titers and titers of three subclasses of IgG were compared in the six mice that died and an equal number of mice from the same groups that lived, and these mice had similar total IgG V-ELISA titers (Table 3). The serum was obtained 1 week prior to challenge. The GMTs for total IgG, IgG2a, and IgG2b of the six mice that died were not statistically different from the mice that lived. However, the GMTs of IgG1 between the survivors and the mice that died were significantly different ($P = 0.0277$) (Table 3).

While spleen weights were used as one means of determining the degree of disease caused by the infection, the postchallenge titer could also be used as an indication of the extent of bacterial replication. The pre- and postchallenge IgG ELISA GMTs were compared for the group challenged s.c. with 1.2×10^6 LD_{50} of the CO92 strain. The titers were statistically different ($P = 0.01$).

DISCUSSION

A previous study (22) demonstrated that intraparenteral immunization with rV combined with incomplete Freund's adjuvant could protect inbred mice against a lethal wild-type *Y. pestis* infection. In this study we extended this finding by demonstrating the efficacy of rV against lethal aerosol infections with $F1^+$ and $F1^-$ strains of *Y. pestis*. Furthermore, this study found that when rV was combined with the human use adjuvant aluminum hydroxide, administered s.c., the antigen was efficacious. In addition, we showed that a reduced, two-dose, immunization schedule provides significant protection. This study was conducted with outbred mice, which may better mimic the genetic variability in the human population.

Previous studies demonstrated a clear role for antibody to the V antigen in passive antibody protection studies (21, 35, 40), though the mechanism by which the V antibody prevents fatal disease in not known. A good IgG antibody response to the rV adsorbed to aluminum hydroxide developed as early as 13 days postimmunization, and titers continued to rise up to the time of challenge on day 58 postimmunization. The predominant IgG subtype was IgG1, followed by IgG2a and IgG2b. This pattern is typical when aluminum hydroxide is used as an adjuvant with various antigens (1, 20). When the total IgG, IgG1, IgG2a, and IgG2b titers of the mice that died were compared to those of survivors, only the IgG1 titers were statistically different. However, with only 6 deaths out of the 98 immunized and challenged mice, additional studies with a suboptimum dose of rV will be needed to correlate V antibody titers with protection, as has been done with the *Y. pestis* F1 antigen (6, 25, 44).

The fact that only 1 spleen out of the 90 tested at 28 days postchallenge contained viable *Y. pestis* cells suggests that immunization with rV is effective for inducing clearance mechanisms for $F1^+$ as well as $F1^-$ organisms from most individuals in an outbred population. The only infection that was not cleared from an immunized mouse resulted from an s.c. challenge with the $F1^-$ C12 strain. $F1^-$ variants have been isolated from animals infected with $F1^+$ strains (2, 9, 43) and have been associated with chronic infections.

In this study, significant increases in group spleen weight were not obtained for all of the infected groups of mice, indicating a variable response to the challenge. Bacteremias, organ titers, or temperatures of the infected mice were not determined in this study but need to be determined to better assess the extent of disease in the immunized, challenged outbred mice. The ability of rV to prevent death and the establishment of a chronic infection in the majority of the surviving mice challenged s.c. or by aerosol indicates the value of this immunogen as part of any subunit vaccine. However, the ability of this immunogen to prevent disease needs to be ascertained.

It is possible to protect mice from a lethal aerosol wild-type *Y. pestis* infection by administering polyclonal, monospecific (37) or IgG1 monoclonal antibodies (2) directed against the *Y. pestis* F1 antigen or by active s.c. immunization with recombinant F1. These experiments demonstrated that immunization at mucosal surfaces is not an absolute requirement for protection against pneumonic plague. This study further supports these findings by demonstrating that s.c. immunization with rV protected all mice against death from a substantial (971 LD_{50} [CO92]) aerosol infection. Whether immunization at mucosal surfaces could enhance the protection afforded by rV will have to await other studies. These results clearly demonstrate for the first time that s.c. immunization with rV can be effective against a *Y. pestis* inhalation challenge.

The efficacy of the current licensed plague vaccine, USP, against pneumonic plague in humans is unknown. While indirect evidence suggests that the vaccine is effective against bubonic plague (10), individuals immunized with this vaccine have still developed pneumonic plague (24). Smith and Packman demonstrated that the plague vaccine USP could elicit marginal protection in mice challenged by an aerosol of wild-

TABLE 3. Mean log reciprocal antibody titers of rV-immunized mice which survived or died from a *Y. pestis* challenge[a]

Outcome	V-ELISA Titer			
	IgG[b]	IgG1[c]	IgG2a[b]	IgG2b[b]
Death ($n = 6$)	5.66 ± 0.17	5.56 ± 0.30	4.06 ± 0.70	4.06 ± 0.48
Survival ($n = 6$)	5.76 ± 0.23	5.91 ± 0.16	4.36 ± 0.44	4.26 ± 0.35

[a] Female Swiss Webster (Hsd:ND4) mice were immunized s.c. with 10 μg of rV adsorbed to an aluminum hydroxide adjuvant (Alhydrogel) on days 0 and 30. Mice were bled 1 week before challenge with a lethal dose of *Y. pestis* C12 or CO92 either by aerosol or s.c. Six mice with similar IgG V-ELISA titers from the respective challenge groups were chosen for the subclass comparison. Log_{10} reciprocal endpoint titers were determined for individual mice and the mean ± standard deviation is presented for each group of animals. No specific rV antibodies were detected in sera of control mice.

[b] Not statistically significant ($P > 0.05$).

[c] $P = 0.0277$, Student's *t* test.

type plague (36). We confirmed this in the mouse aerosol model with the $F1^+$ CO92 strain (4, 31), and we showed that the plague vaccine USP does not offer protection against the $F1^-$ C12 strain (18). The lack of significant protection could be due to the subimmunogenic amount of V in the plague vaccine USP. Chen et al. (11) were unable to detect antibodies to the V protein by using an Ouchterlony test. We did not detect V antibodies by ELISA in mice that received two s.c. or intramuscular immunizations with plague vaccine USP (18, 45). We were not able to detect the V protein in the plague vaccine USP with Western blots by using monospecific V antiserum and enhanced chemiluminescence (3).

Y. pestis strains expressing the F1 antigen are the usual phenotype encountered in nature. Therefore, the F1 antigen should be included in any vaccine, and it is the principal immunogen in the current plague vaccine, USP. Recently, Williamson et al. (45) demonstrated the feasibility and efficacy of a subunit vaccine consisting of F1 and V antigens combined with Freund's incomplete adjuvant, which offered better protection than either antigen alone against an s.c. challenge. In subsequent work, we also showed that an F1-V fusion protein protects against an aerosol challenge (18). These results suggest that a subunit vaccine consisting of at least the F1 and V antigens should provide better protection against pneumonic plague than that shown with V alone, as in this study.

In conclusion, we demonstrated that rV formulated for human use can protect against pneumonic as well as bubonic plague. This plague vaccine significantly protected mice against pneumonic plague caused by either $F1^+$ or $F1^-$ *Y. pestis.*

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