SHORT- AND LONG-TERM EFFICACY OF SINGLE-DOSE SUBUNIT VACCINES AGAINST YERSINIA PESTIS IN MICE

GEORGE W. ANDERSON JR, DAVID G. HEATH, CHRISTOPHER R. BOLT, SUSAN L. WELKOS, AND ARTHUR M. FRIEDLANDER

Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Maryland

Abstract. A single, subcutaneous, 30-µg dose of either a combination of the Yersinia pestis proteins F1+V or a F1-V fusion protein adsorbed to the adjuvant aluminum hydroxide, protected Hsd:ND4 mice for one year against pneumonic plague. The recombinant F1+V vaccine provided significant protection as early as day 14 postimmunization. The current Plague Vaccine USP in a single 0.2-ml dose did not provide significant protection in this mouse model. Antibody titers to F1 and V peaked at approximately 5–12 weeks postimmunization and were still detectable one year later. These F1 and V subunit vaccines may offer effective long-term immunity with a reduced dosage schedule when compared with the presently licensed, formalin-killed, whole-cell vaccine.

Plague caused by the gram-negative bacterium Yersinia pestis still represents a serious public health threat in various regions of the world, as evidenced by the announcement of a plague outbreak in India in 1994,1, 2 which severely disrupted the commerce and the public health systems.3 Even though live and killed vaccines were available for human use to prevent plague, the reactogenicity, long immunization schedule, requirement for frequent boosts, general low risk of infection, and lack of demonstrated efficacy against pneumonic plague limited their use before or during the outbreak.4 Use of a single-dose vaccine with low reactogenicity and rapid onset of protection against the bubonic and pneumonic forms of the disease could have possibly reduced the level of public health concern and economic impact on India.

Two Y. pestis proteins, F1 and V, are known to be effective immunogens.5–9 The F1 capsular protein, unique to Y. pestis, has long been considered the principle protective antigen in the Plague Vaccine USP and has been used for Y. pestis identification and disease diagnosis.10 Several studies have been unable to detect a measurable V antibody response after immunization with the Plague Vaccine USP.11, 12

The 15.5-kD F1 protein encoded on the 100-kb plasmid pFra is an important but not essential virulence factor with anti-phagocytic activity.10 Clinical studies conducted from 1945 to 1952 with milligram doses of soluble F1 examined the reactogenicity and the nature of the protective antibody produced.13, 14 These immunizations, most with adjuvants, induced levels of F1 antibodies that were thought to be protective based on the mouse protection index.13 However, these larger doses were very reactogenic in some human volunteers.14 Recent protection studies in mice15, 16 with approximately 100-fold less F1 protein than used in the human trials, provided significant protection against bubonic and pneumonic plague. The F1 in the mouse was adsorbed to the human use adjuvant aluminum hydroxide.

The V protein, a 37-kD protein encoded on the 70–75 kb LCR plasmid, is also a virulence factor associated with protective immunity, modulation of cytokines affecting the inflammatory response, and regulation of the low calcium response.6, 9, 17 The V protein is common to the three human Yersinia pathogens. The V proteins from different strains of Y. enterocolitica differ in their ability to induce cross-protection due to variations in their amino acid sequences.18 Differences in cross-protection have not been shown for the V protein among Y. pestis strains, although few strains have been tested.

Recent studies have shown that multiple doses of recombinant subunit vaccines consisting of either F1, V, F1 and V, or an F1-V fusion protein can protect mice against bubonic and/or pneumonic plague caused by wild-type Y. pestis.12, 15, 16, 19, 20 The F1 and V immunogens are also efficacious against plague caused by an F1- strain.16, 20 The F1+V combination vaccines are superior to either F1 or V administered individually.12, 13 The above studies used similar immunization schedules, either two doses given on days 0 and 28, or three doses given on days 0, 14, and 28. None of these studies examined the efficacy of a single dose, reported the time required to induce a protective response, evaluated the number of vaccine doses required for protection, or examined the long-term antibody response and protection. In this study, we examined several doses of either F1 or V immunogens individually against challenge with wild-type Y. pestis, and a combination F1+V subunit vaccine against challenge with wild-type and an F1- virulent isogenic strain. We then compared the efficacy of a single-dose of this vaccine to a F1-V fusion protein vaccine over one year against challenge with the fully virulent wild-type CO92 strain of Y. pestis.

MATERIALS AND METHODS

Animal immunizations. Female 8–9-week-old outbred Swiss Webster mice (Hsd:ND4) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Experiments with animals were conducted according to the principles set forth in the Guide for the Care and Use of Animals.21 Mice were given food and water ad libitum. Mice were immunized subcutaneously with 0.2-ml aliquots of recombinant F1, V, F1+V, or F1-V fusion protein or the licensed Plague Vaccine USP (Greer Laboratories, Inc., Lenoir, NC). The Y. pestis antigens were adsorbed to an aluminum hydroxide adjuvant (1.3% Alhydrogel, lot no. 4023; Superfos Biosector, Vedbaek, Denmark). While various concentrations of recombinant proteins (rV, rF1, or rF1+V) were used, the amount of adjuvant was held constant at 0.19 mg of aluminum/0.2-ml dose. Transponders with unique identification numbers (BioMedic Data Systems, Inc.; Seaford, DE) were injected subcutaneously near the scruff of the neck one week before...
immunization so that the responses of individual mice to immunization and challenge could be determined.\textsuperscript{22} Protein content of the immunization doses was determined by the bicinchoninic acid-Lowry method with bovine serum albumin as a standard (Pierce, Rockford, IL). Endotoxin levels were determined with the Limulus amoeocyte lysate assay (Sigma; St. Louis, MO) (1 endotoxin unit = 0.1 ng of Escherichia coli 055:B5 lipopolysaccharide standard).

**Recombinant V (rV) protein.** Expression and purification of the recombinant V (rV) protein will be described in detail elsewhere.\textsuperscript{23} Briefly, to express and purify V, the LcrGVH operon\textsuperscript{14} was ligated into pBluescript and cloned into E. coli DH5α (Stratagene, La Jolla, CA) after a polymerase chain reaction (PCR) using the Y. pestis Antigua strain Pgm-plasmid DNA. The cloned LcrGVH operon then served as template DNA in a PCR to isolate the V antigen structural gene. The V antigen PCR product was ligated directly into the plasmid PCRII (Invitrogen; San Diego, CA) to transform E. coli DH5α. Plasmid DNA purified from this clone was digested with Nde I and the V antigen-containing fragment was then ligated into pET15b (Novagen, Madison, WI). The V antigen protein with a fused 20 residue N-terminal peptide segment (of which six were contiguous histidines) was subsequently expressed in E. coli BL21(DE3) (Novagen). The protein was partially purified using Ni\textsuperscript{2+} chelating resin under nondenaturing conditions (Novagen), then more highly purified by ion-exchange chromatography using Whatman (Fairfield, NJ) DE-52 cellulose. The 20 residue N-terminal peptide segment was not removed before immunization. This preparation contained 0.13 endotoxin units/mg.

**Recombinant F1 (rF1) protein.** The rF1 antigen was expressed in E. coli HB 101 (PBRF1) and purified as previously described from the culture supernatant.\textsuperscript{15} This preparation contained 13 endotoxin units/mg of F1.

**Recombinant F1-V (rF1-V) protein.** Expression and purification of recombinant F1-V (rF1-V) will be described in detail elsewhere.\textsuperscript{23} Briefly, to isolate the F1 structural gene minus its stop codon, plasmid pYPR1 (kindly provided by T. Schwan, Rocky Mountain Laboratories, Hamilton, MT) containing the F1 operon\textsuperscript{7} was used as template DNA in a PCR. The V antigen gene was then isolated by PCR with plasmid DNA from a pigmentation-negative (Pgm-) derivative of the Y. pestis Antigua strain. The purified F1 PCR product was then digested with Nde I and Eco RI while the V antigen purified PCR product was digested with Eco RI and Bam HI. Both restricted fragments were then ligated to the Nde I- and Bam HI-digested expression vector pET 19b and used to transform E. coli strain BLR (Novagen) to create plasmid pF1V. The final protein consisted of an amino terminal 10 histidines and an enterokinase site from pET 19b followed by the F1-V protein. The F1 portion consisted of 170 amino acids followed by two amino acids, asparagine and glutamine (encoded by the Eco RI site), and the entire sequence of the V antigen. The F1-V protein, therefore, had 521 amino acids with a predicted molecular mass of 57,926 daltons. The amino-terminal polyhistidine residues were not removed before immunization.

The F1-V fusion protein expressed from pET 19b was isolated with 6 M urea as recommended (Novagen). Residual endotoxin was removed by passing F1-V over an endotoxin-removing gel column (Pierce). After purification through the column, F1-V contained 125 endotoxin units/mg of protein.

**Measurement of serum antibody titer.** Blood was collected from the retro-orbital sinus one week before challenge or by cardiac puncture for terminal bleeds from mice anesthetized with a cocktail containing xylazine, 5 mg/kg (Xyla-Ject; Phoenix Pharmaceutical, Inc. St. Joseph, MO), acetylpromazine, 0.83 mg/kg (Fermenta Animal Health Co., Kansas City, MO), and ketamine hydrochloride, 67 mg/kg (Ketamine; Phoenix Pharmaceutical, Inc.) administered intramuscularly. Total IgG serum antibody titers to the F1 or V antigens were determined by using a modification of an ELISA as previously described.\textsuperscript{25} The endpoint titer was defined as the highest test serum dilution giving a reading ≥ 0.1 optical density units after subtraction of background from the wells without antigen. The results obtained are expressed as the geometric mean titer (reciprocal endpoint titers). In the case of individual titers < 640, the initial dilution, the convention of using half of this value for statistical purposes, was used.

**Animal challenge with Y. pestis.** Groups of immunized or control mice were challenged subcutaneously with 0.2-ml aliquots of Y. pestis wild-type CO92\textsuperscript{29} or the isogenic F1-C12\textsuperscript{27} cells or with a small-particle aerosol of CO92 and C12 as previously described.\textsuperscript{25} Mice were exposed to an aerosol in a nose-only exposure system contained within a Class III biological safety cabinet. A subcutaneous challenge was used for antigen dose-response measurements because the infectious dose delivered could be more precisely controlled than with the aerosol challenge. All infectious experiments were conducted in a facility meeting Biological Safety Level 3 standards.\textsuperscript{26}

**Bacteria.** The propagation, storage, and characteristics of the Y. pestis strains have been previously described.\textsuperscript{27, 28} The wild-type CO92\textsuperscript{26} and isogenic F1-C12\textsuperscript{27} strains have been previously described.\textsuperscript{27, 28} The subcutaneous 50% lethal doses (LD\textsubscript{50}) for adult mice were 1.9 and 9.1 colony-forming units (CFU) for CO92 and C12, respectively, and the aerosol LD\textsubscript{50} of the CO92 strain for adult mice was 2 × 10\textsuperscript{4} CFU, all calculated at day 14. Mice were observed daily for 28 days at which time the survivors were bled for antibody determination. The spleens were removed aseptically, weighed, homogenized, and assayed for viable Y. pestis cells on sheep blood agar plates.

**Statistical analysis.** The Fisher exact test and Student’s t-test were used to compare the various treatment groups at the 0.05 level of probability.

**RESULTS**

**Dose response of either F1 or V.** Before using combinations of F1+V or the F1-V fusion protein, we determined the dose response to the individual proteins. The antibody response and protection afforded by a single subcutaneous dose of F1 absorbed to aluminum hydroxide against the wild-type CO92 strain at day 46 postimmunization is shown in Table 1. Only four of the 10 mice examined developed a measurable F1 ELISA titer to a 0.1 µg dose of F1 while 10 of 10 developed a detectable response to a 1.0 µg dose. However, a dose of F1 ≥ 10 µg was required to give the maximum protective response to this challenge. Immunizing doses > 10 µg induced a higher F1 antibody response, but neither protection nor mean time to death was increased.
This indicated that a 10 μg dose of F1 was probably the minimum single dose for immunization with F1 alone.

Table 2 depicts the results of immunizing mice with a single dose of the V protein ranging from 0.1 to 40 μg adsorbed to aluminum hydroxide. Ten of 10 mice examined developed an anti-V antibody titer to 0.1 μg of the V protein. As with F1, the highest dose, 40 μg, induced the highest antibody response, but it was not possible to determine if 20 or 40 μg was more protective against the F1- mutant of CO92, strain C12. These data suggest that 20 μg of V is probably the minimum single dose for immunization.

**Dose response of an F1 and V combination vaccine.**

The results of immunizing mice with various single doses of a combination F1 and V vaccine are presented in Table 3. The ratio of F1 to V on a weight basis was 1:2, the approximate ratio of the F1 and V portions of the F1-V fusion protein. Vaccine doses were selected to bracket doses that were previously shown to be protective. These doses provided an estimate of the minimum protective immunogen dose and an acceptable immunizing dose, but not a maximum-tolerated dose. We tried to choose challenge doses of *Y. pestis* CO92 and C12 strains that would delineate non-protective and protective immunogen doses. The lowest dose of F1+V induced IgG anti-F1 and anti-V antibody responses in only four of nine mice for F1, but in nine of nine mice for V. This dose was sufficient to protect 67% of the mice against lethal infection with CO92, however, all of the mice challenged with C12 died. The geometric mean anti-V antibody titer was 37,098. This suggests that low IgG F1 titers are protective, while 40-fold higher V antibody titers are not protective. As little as 0.5 μg of rF1 and 1.0 μg of rV provided > 80% protection against CO92 and C12 challenge, with all mice producing detectable antibody to F1 and V. In general, a higher immunizing dose induced higher titers of F1 and V antibodies, but the challenge doses of CO92 and C12 selected could not distinguish significant differences in protection with the higher immunizing doses.

To determine the necessity of the adjuvant, the highest immunizing dose of F1+V was also evaluated without the adjuvant, aluminum hydroxide. In this case, both F1 and V antibodies were induced in 10 of the 10 mice; however, the antibody responses were approximately 10-fold less than the equivalent group with adjuvant. Protection of mice immunized with an F1+V dose without the adjuvant was significantly decreased (P ≤ 0.003) compared with the same dose with adjuvant, when mice were challenged with the CO92 or C12 strains.

We also tested the response of mice to a single 0.2 ml dose of the Plague Vaccine USP. This dose, which is 20% of the initial human dose, only partially protected mice against the CO92 strain and failed to protect against the F1-strain, C12. In support of previous observations, no V antibody titers were detected before challenge of mice immunized with the Plague Vaccine USP, so protection was not expected. Here, we determined how quickly a protective response developed in mice and how long this response could be sustained. The results of immunizing with a single 30-μg dose of either F1+V or the F1-V fusion protein adsorbed to Alhydrogel are compared in Table 4. As early as 14 days post-immunization, the F1+V combination induced significant protection (P = 0.006) against an aerosol challenge of the wild-type CO92 strain when compared with the control group. The protection observed for the F1-V fusion vaccine group at day 14 (27%) did not differ significantly from the control. However, by day 42 postimmunization, protection was indistinguishable for either F1+V or F1-V vaccine and protection continued for one year, the last time point tested.

**Long-term protection induced by F1+V or F1-V immunogens.** A previous study determined that multiple doses of an F1-V vaccine could protect against an aerosol challenge from either the wild-type CO92 or the F1- C12 strain. Here, we determined how quickly a protective response developed in mice and how long this response could be sustained. The results of immunizing with a single 30-μg dose of either F1+V or the F1-V fusion protein adsorbed to Alhydrogel are compared in Table 4. As early as 14 days post-immunization, the F1+V combination induced significant protection (P = 0.006) against an aerosol challenge of the wild-type CO92 strain when compared with the control group. The protection observed for the F1-V fusion vaccine group at day 14 (27%) did not differ significantly from the control. However, by day 42 postimmunization, protection was indistinguishable for either F1+V or F1-V vaccine and protection continued for one year, the last time point tested. When the pre- and post-challenge F1 and V ELISA titers were examined for the group challenged at one year, there was a 2–3-fold increase in the geometric mean titers after challenge for either vaccine. Thus, the animals became infected, but replication was probably not extensive. Additionally, spleen weights of the survivors at the one-year challenge point were not different than those of the noninfected control mice. No viable *Y. pestis* cells were detected in spleen cell homogenates of the survivors.

A larger study is needed to confirm whether F1+V is superior to the F1-V fusion protein at the early time point. There was no statistical difference in the number of survivors between the F1+V or the F1-V fusion protein vaccine.
groups (8 of 14 versus 4 of 15; \( P = 0.139 \)). The F1 antibody titer to the F1+V vaccine was higher than the response to either F1-V vaccine or Plague Vaccine USP (Figure 1A). The antibody titers to the V protein from animals vaccinated with either the F1+V or F1-V vaccine were similar, undetectable at day 7, but approximately one million at day 35 postimmunization (Figure 1B). Low V antibody titers were not protective, as shown in Tables 2 and 3 with the Y. pestis F1- strain, so the protection at day 14 may have been due to the F1 immunogen. However, we did not test this directly in this experiment. Conversely, there was a detectable F1 ELISA titer, but no detectable V titer from immunization with the Plague Vaccine USP. The protection induced in mice from a 0.2-ml subcutaneous dose of the Plague Vaccine USP was never significant when compared with the controls at any time point.

The F1 antibody titer has been used to judge the immune status of vaccinees receiving the Plague Vaccine USP.\(^{a}\) Although protection is the ultimate test of any vaccine, measuring the antibody response to a single dose of vaccine over time can provide a good indication of when a booster dose might be recommended, especially in the case of plague, in which antibody to both F1 and V has proven to be protective. In this study, as depicted in Figure 1A, the peak ELISA antibody titer for F1 was between five and 12 weeks postimmunization, while the antibody response to V appeared to peak at five weeks postimmunization (Figure 1B). The F1 and V antibody responses decreased after this time, but were still detectable at one-year postimmunization, the last challenge time point.

### DISCUSSION

In this study, a single-dose, combination F1+V and a F1-V fusion protein subunit vaccine was shown to induce a rapid immune response. The combination F1+V vaccine significantly protected mice as early as 14 days postimmunization against a wild-type Y. pestis aerosol challenge. Such rapid protection by a subunit vaccine against pneumonic plague has not been previously shown. These results suggest that it may be possible in the future to immunize people during an outbreak like the one in India\(^{2}\) and induce significant protection quickly. While live attenuated Y. pestis vaccines have been used in the past to immunize people during an epidemic,\(^{31}\) reactogenicity has been an issue. Additionally, with a subunit vaccine, antibiotics can be administered prophylactically to at-risk personnel, while they develop active immunity from immunization. This is not possible with a live-attenuated vaccine.

This study demonstrated that low V ELISA antibody titers do not protect against an F1- Y. pestis strain. Although we did not formally test this with the F1+V vaccine, the early protection observed against an aerosol exposure to the wild-type Y. pestis at day 14 postimmunization was probably due to the antibody response against F1. Our data suggest that even though the V immunogen appears to offer greater protection than F1 when the response is fully developed, F1 may induce a more rapid protective response at the doses tested. Therefore, a vaccine formulated for rapid protection...
that study, a total of 3–12 mg of the F1 protein were administered over a 14-day period. A number of studies have shown that increasing the interval between doses in a primary series induces a superior immune response; however, it is difficult to extrapolate data from experimental animals to humans. While most of the human volunteers received the F1 protein without an adjuvant, our use of the adjuvant aluminum hydroxide increased the antibody response to the F1 and V antigens and the level of protection to both wild-type and F1- Y. pestis strains. This suggests that it may be possible to reduce the amount of the immunogen in future clinical studies.

The long-term antibody response to a single dose of either the F1+V or the F1-V vaccine, suggests that recent multi-dose immunization schedules administering two or three doses of recombinant plague vaccines to mice within four weeks may not be optimal. Booster doses were given while the primary antibody response was still increasing. There are data for inactivated hepatitis A vaccines indicating that delaying the booster dose until the primary response is decreasing may offer a more optimal schedule for induction of long-term, persistent immune memory. In adults, a primary immunization with the hepatitis A vaccine followed by a booster dose at six or 12 months is projected to offer protection for > 20 years. The recommended primary immunization schedule for the current Plague Vaccine USP is a 1.0-ml dose intramuscularly followed by a 0.2-ml dose at four weeks and six months. An additional 0.2-ml booster dose at six-month intervals is recommended for persons at risk. Booster doses at 1–2-year intervals may be appropriate for persons who have received three or more booster doses at six-month intervals. While the current study found that a single dose of the Plague Vaccine USP equivalent to 20% of the initial human dose was not efficacious in mice against an aerosol challenge of wild-type Y. pestis, immunizing African green monkeys with the recommended human dose and schedule also failed to protect against a wild-type Y. pestis aerosol challenge (Pitt MLM, USAMRIID, unpublished data). This suggests that the lack of protection against aerosol exposure was not due solely to the reduced dose and schedule in the mice.

While a single dose of F1+V or F1-V provided excellent protection against a lethal aerosol challenge of Y. pestis for one year, a booster dose should offer better immunity than just a priming dose. The antibody titer to F1 correlates with protection and antibodies to passively protect against lethal Y. pestis infection. Therefore, a booster dose given before one-year postimmunization would probably be recommended, but higher challenge doses will be required to demonstrate whether it is necessary.

In summary, a single dose of a vaccine consisting of the F1 and V antigens formulated with human use adjuvant protected mice against pneumonic plague as early as two weeks postimmunization and for periods up to one year. In contrast to the current Plague Vaccine USP, these vaccines offer the possibility of using smaller vaccine doses, extending the interval between immunizations, reducing reactogenicity, and protecting from pneumonic plague.

Acknowledgments: We thank Kevin Gianunzio and Aristotle Archer for technical assistance. Additionally, we are most grateful to Dr.

Figure 1. Geometric mean ELISA titers of Hsd:ND4 mice immunized subcutaneously with 30 μg of rF1-V or 10 μg of rF1 + 20 μg of rV adsorbed to aluminum hydroxide or 0.2 ml of the Plague Vaccine USP (n ≤ 15). The initial dilution was 1:640. A, F1 ELISA titers, B, V ELISA titers.

may contain an amount of F1 closer to the maximum tolerated dose, than a minimum protective dose.

Recent studies show that vaccines containing F1+V are more effective than immunizing with either F1 or V alone against wild-type F1+ Y. pestis strains. These studies found that less F1+V was needed to induce the same level of protection obtained with either F1 or V alone. Similar results have been obtained with the F1-V fusion protein. A plague vaccine containing multiple immunogens has been suggested to provide protection against F1- strains. A multi-immunogen vaccine may also offer at least partial protection to individuals who are nonresponders to the F1 component of the current Plague Vaccine USP. We estimate that the 30-μg dose of the F1+V vaccine used in this study, is approximately 20-fold greater than the minimum protective dose of F1+V against F1+ and F1- strains of Y. pestis for a mouse. This dose is substantially less than below the 1–4-mg doses of purified, soluble F1 that were administered to human 306 volunteers and found to be too reactogenic. In

In

against wild-type F1 más efectivo que inmunizarse con F1 o V solos.

ELISA titers,
Margaret Pitt and Ralph Tammarinello for assistance in performing the aerosol challenges.

Financial support: This study was supported by the U.S. Army Medical Research and Materiel Command.

Disclaimer: The opinions or assertions contained in this manuscript are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the United States Army Medical Research Institute of Infectious Diseases.

Authors' addresses: George W. Anderson, Jr., Christopher R. Bolt, Susan L. Welkos, and Arthur M. Friedlander, Bacteriology Division, USAMRIID, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5011. David G. Heath, Landstuhl Regional Medical Center, CMR 402, Box 1290, APO-AE 09180-3460.

Reprint requests: George W. Anderson, Jr., Bacteriology Division, USAMRIID, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5011.

REFERENCES


