

## Biosafety Level 2 Model of Pneumonic Plague and Protection Studies with F1 and Psa<sup>▽</sup>

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**Attenuated *Yersinia pestis* *pgm* strains, such as KIM5, lack the siderophore yersiniabactin. Strain KIM5 does not induce significant pneumonia when delivered intranasally. In this study, mice were found to develop pneumonia after intranasal challenge with strain KIM5 when they were injected intraperitoneally with iron dextran, though not with iron sulfate. KIM5-infected mice treated daily with 4 mg iron dextran died in 3 days with severe pneumonia. Pneumonia was less severe if 4 mg iron dextran was administered only once before infection. The best-studied experimental vaccine against plague currently consists of the *Yersinia pestis* capsular antigen F1 and the type 3 secreted protein LcrV. The F1 antigen was shown to be protective against KIM5 infections in mice administered iron dextran doses leading to light or severe pneumonia, supporting the use of an iron dextran-treated model of pneumonic plague. Since F1 has been reported to be incompletely protective in some primates, and bacterial isolates lacking F1 are still virulent, there has been considerable interest in identifying additional protective subunit immunogens. Here we showed that the highly conserved Psa fimbriae of *Y. pestis* (also called pH 6 antigen) are expressed in murine organs after infection through the respiratory tract. Studies with iron dextran-treated mice showed that vaccination with the Psa fimbrial protein together with an adjuvant afforded incomplete but significant protection in the mouse model described. Therefore, further investigations to fully characterize the protective properties of the Psa fimbriae are warranted.**

*Yersinia pestis*, a Gram-negative bacterium belonging to the family *Enterobacteriaceae*, is the causative agent of plague, a disease that affects a variety of mammalian hosts and that can be transmitted by various arthropod vectors. Humans are susceptible to *Y. pestis*, whether transmitted by aerosol or by infected fleas, causing the highly lethal pneumonic plague or bubonic plague, respectively (53). Aerosol infections by *Y. pestis* remain a concern for public health (12). The only currently available tools for combating *Y. pestis* in the United States are antibiotics, since manufacture of the previously licensed formalin-killed *Y. pestis* vaccine that was used by the U.S. military during the Vietnam War (41) stopped in 1998. Although effective against bubonic plague, this vaccine was not efficiently protective against pneumonic plague and caused significant side effects (11). The emergence of multiple antimicrobial resistance in *Y. pestis* (72) and the lack of a safe and effective vaccine have been the impetus for the search for new inhibitory drugs as well as a better vaccine (48, 49).

*Y. pestis* has been classified as a Category A select agent (8)

requiring special precautions, particularly because of the risk of pneumonic plague due to bacterial aerosolization. One of the major impediments to studying the biology and pathogenesis of *Y. pestis* is that many laboratories do not have access to the required biosafety level 3 (BSL-3) facilities and therefore must work with attenuated strains that were excluded from the CDC select-agents list. Such strains lack either the *pgm* locus (e.g., KIM5) or the virulence plasmid pCD1 (51). A major virulence component of the *pgm* locus is the yersiniabactin (Ybt siderophore)-dependent iron transport system, which is essential for plague infection from peripheral sites (52). The pCD1 plasmid encodes a type III secretion system (T3SS, or Yops regulon) that, upon contact with host cells (or in low calcium concentrations *in vitro*), produces an elaborate injection machinery used to transfer a set of effector proteins with potent antiphagocytic and/or anti-inflammatory effects (2, 67) directly to the cytosol of the eukaryotic cell. Mutants lacking *pgm* or pCD1 are safer to manipulate, allowing experimental studies to be carried out in BSL-2 laboratories. However, the disease progression elicited by these strains does not recapitulate that observed with fully virulent strains (44), and in comparison to the wild-type strain, these strains are highly attenuated by any natural route of infection. Specifically, the  $\Delta$ *pgm* strain KIM5 is attenuated when given subcutaneously (s.c.) (52). Similarly, another  $\Delta$ *pgm* derivative of the virulent strain KIM, strain D27 (KIM D27), was unable to grow to high numbers in the lungs of intranasally infected mice, suggesting that bacterial growth was contained by the host's innate immune system. Moreover, these mice did not develop significant pneumonia, as they would if infected with wild-type *Y. pestis*

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strains (33). However, strain KIM D27 was still able to spread and grow in spleens and livers. This suggested that the iron uptake system of the *pgm* locus was needed to counteract iron restriction in the lungs. Interestingly, the results of earlier studies suggested that parenteral administration of iron sulfate to mice could bypass the need for yersiniabactin, rendering a parenterally administered *Y. pestis*  $\Delta$ *pgm* mutant fully virulent (10). However, a recent study showed that the use of the same approach to induce pneumonic plague after intranasal (i.n.) administration of *pgm* strain KIM D27 was not successful (33). This study also highlighted the toxicity of ferrous chloride, limiting administration doses to 0.5 mg per mouse. The latter result was consistent with the early toxicity data for inorganic iron at 30 to 60 mg/kg of body weight (26).

The toxicity problem of inorganic iron and the need for iron treatments for various medical conditions led to the development of less-toxic colloids consisting of various carbohydrates with ferric (oxy)hydroxide (16). In agreement with the results of earlier studies with inorganic iron, intraperitoneal (i.p.) administration of colloidal iron to mice subsequently infected subcutaneously with a *Y. pestis* *pgm* strain resulted in death, with histological lesions in the livers and spleens resembling those resulting from infection with a virulent strain (68). A variety of more-recent models of bacterial infections were developed by taking advantage of the extremely low toxicity of the colloid iron dextran, administrations of which in the milligram range were shown to be nontoxic (29, 62, 75). Based on the success of these models, we undertook experiments to determine whether the administration of high doses of iron dextran would permit *Y. pestis* to grow in the lungs of i.n. infected mice and cause local inflammation. Here we describe the development of a BSL-2 pneumonic plague model in mice based on the administration of iron dextran. In contrast to the negative results obtained with iron chloride, the use of iron dextran has allowed us to bypass toxicity problems and to take advantage of its favorable pharmacokinetic properties, such as slower clearance and a longer half-life for iron than those with other colloidal or inorganic iron preparations (16).

Our model was tested by confirming the expected protective property of F1 in pneumonic plague. We also used the model to evaluate the potential protective property of the Psa fimbriae (pH 6 antigen). Both F1 and Psa are surface structures that share the structural properties of homopolymeric fimbriae, each formed by a protein subunit that requires its own chaperone and usher proteins for export. Structural studies have classified F1 and Psa within a family of fimbrial polyadhesins assembled by FGL chaperones (for their long F1 and G1  $\beta$ -strands) (76). The F1 capsular antigen is an efficient immunogen that is protective by itself in a murine model of pneumonic plague with F1<sup>+</sup> strains (4). The protective property of the F1 immunogen can be attributed in great part to its polymeric structure and *in vivo* surface exposure. Similarly, mixtures of fimbriae and surface-exposed adhesins of other pathogens have been used successfully as commercial vaccine components for both humans and animals (17, 43, 54). Surface-expressed antigens such as F1 induce opsonizing and/or antivirulence antibodies, and such antibodies by themselves are protective (28). A role for opsonization by antibodies directed toward *Y. pestis* surface antigens is suggested by studies that showed that neutrophil depletion (15) or inhibition of cyto-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic	Source or reference
Strains		
<i>E. coli</i>		
BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) dcm gal</i> (DE3)	Novagen (Madison, WI)
<i>Y. pestis</i>		
KIM5	pCD1 <sup>+</sup> (LCR <sup>+</sup> ) <i>pgm</i> (Pgm <sup>-</sup> )	23
KIM6	KIM5 pCD1 <sup>-</sup> (LCR <sup>-</sup> )	23
DSY24	KIM5 $\Delta$ <i>caf::blaM</i>	This study
DSY39	KIM5 $\Delta$ <i>caf::blaM</i> $\Delta$ <i>psa::aphA</i>	This study
Plasmids		
pCS267	pBR322- <i>psaABC</i>	37
pCS274	pET16- <i>cafIM-cafIA-cafI</i>	37
pDMS167	pBR322 <i>fas</i> (987P <sup>+</sup> )	59

kines that boost the antimicrobial activity of macrophages (18, 50) interfered with antibody-mediated protection. Properties of Psa similar to those of F1, such as its polymeric structure and bacterial surface location, make it an attractive vaccine candidate. Therefore, we evaluated Psa for its *in vivo* expression, immunogenicity, and protective properties by using the pneumonic plague model that we developed.

#### MATERIALS AND METHODS

**Reagents and antibodies.** All bacteriological medium components and Freund's adjuvants were from Difco (BD Diagnostics, NJ). Iron dextran (ferric hydroxide dextran complex) was from Sigma-Aldrich (St. Louis, MO). Aluminum hydroxide (Alhydrogel) was obtained from Accurate Chemical (Westbury, NY). The specific rabbit antisera against the *Y. pestis* Psa and F1 proteins have been described previously (37).

**Bacterial strains and plasmid constructs.** The *Escherichia coli* and *Y. pestis* strains and the plasmids used in this study are listed in Table 1. *E. coli* was routinely grown at 37°C in Luria-Bertani (LB) medium, supplemented with ampicillin (200  $\mu$ g/ml) when appropriate. *Y. pestis* strains were grown overnight in brain heart infusion broth (BHI), at 26°C, diluted 1:10 in fresh BHI containing 2.5 mM CaCl<sub>2</sub>, buffered to pH 6.0 with 0.1 M MES (morpholineethanesulfonic acid), and cultured overnight at 37°C. Bacterial counts were determined on TB (13 g tryptone broth, 2.5 g NaCl, and 3.0 g Bacto beef extract paste in 1 liter) agar plates. *Y. pestis* KIM5 mutants DSY24 and DSY39 were constructed by allelic exchange, as described previously for the same mutations prepared in *Y. pestis* KIM6 (37). Maintenance of the KIM5 plasmids in the mutants was checked by agarose gel electrophoresis.

**Isolation of fimbriae.** Recombinant Psa, F1, or 987P fimbriae were expressed on the bacterial surface of *E. coli* BL21(DE3) transformed with plasmid pCS267, pCS274, or pDMS167, respectively (Table 1). The surface-expressed proteins were isolated by heat extraction (20, 37) using the following modifications. For the Psa preparation, bacteria were inoculated in LB medium supplemented with ampicillin and were grown overnight at 37°C. Fresh LB medium was subsequently inoculated with 1% of this culture, which was then grown at 37°C for 5 to 7 h (to an optical density at 600 nm [OD<sub>600</sub>] of ~0.8). The bacteria were harvested by centrifugation at 4,000  $\times$  g for 15 min. Next, the cell pellets were suspended in 0.5 mM Tris-HCl (pH 7.4)–75 mM NaCl and were treated at 58°C for 30 min. After centrifugation, ammonium sulfate was added to the supernatant or to the spent medium to 0.5% saturation, and the two fractions were incubated overnight on ice. The precipitated proteins were separated by centrifugation and shown to contain mainly contaminants. The supernatants from the bacterial surface and the spent medium were then subjected to a second precipitation step by addition of a saturated ammonium sulfate solution to reach 30% saturation. Proteins that precipitated overnight at 4°C were collected by centrifugation at 10,000  $\times$  g for 30 min and were dialyzed extensively against distilled water. F1 and 987P preparations were made essentially as described above, with the following minor modifications. F1 expression was induced by addition of 0.25 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) to the bacterial culture (OD<sub>600</sub> ~0.5) and incubation for 2 h at 37°C. The 987P fimbriae were prepared directly from overnight cultures. The heat treatments to isolate F1 and 987P were done at 60°C and 62°C, respectively. The final precipitation step for 987P prep-

aration used a 50% saturated ammonium sulfate solution. The protein concentrations of the fimbrial preparations were determined to be 2.0 to 2.5 mg/liter of bacterial culture for Psa, 2.8 to 4.0 mg/liter for F1, and 3.0 to 3.5 mg/liter for 987P (39). The purity of the proteins (>95%) was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The fimbrial preparations were further purified by affinity chromatography with immobilized polymyxin B to remove excess endotoxin. For this purpose, the proteins (4 mg per 2 ml endotoxin-free distilled water) were passed several times through columns of 2 ml Detoxi-Gel (Pierce), as recommended by the manufacturer. The endotoxin levels of the purified proteins (<0.53 endotoxin units [EU]/dose of 40 µg antigen, corresponding to LcrV and F1 doses administered in a human clinical trial) were significantly below the FDA acceptable levels (350 EU/dose for a 70-kg human), as determined by the *Limulus* amoebocyte lysate assay (Lonza Walkersville Inc., MD) using *E. coli* 0111:B4 lipopolysaccharide (LPS) as a standard (6, 74).

**Infection of mice.** Six- to 8-week-old female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed by following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC), and animals were provided food and fresh water *ad libitum* during the experiment. Bacteria were grown at 37°C and were washed in sterile phosphate-buffered saline (PBS). Numbers of CFU inoculated were determined by plating serial dilutions onto TB agar plates (21). Mice were anesthetized with ketamine and xylazine (100 and 10 mg/kg of body weight) *i.p.* and were inoculated by the *i.n.* route with 20 µl of *Y. pestis* in PBS. In order to set the conditions for iron supplementation in mice, groups of five to eight mice received 1 to 10 mg iron (formulated as iron dextran) *i.p.* 2 to 3 h before *i.n.* inoculation of  $1 \times 10^5$  CFU of *Y. pestis* strain KIM5 or KIM6. In some experiments, 0.75 to 2.0 mg iron dextran was delivered *i.n.* 3 h after the bacterial inoculation. Where indicated, the *i.p.* iron dextran was administered once a day during the course of the experiment. Mice were monitored for survival twice daily for 10 days. For experiments to determine the 50% lethal dose (LD<sub>50</sub>), groups of 10 mice were infected intranasally with serial 10-fold dilutions of *Y. pestis* strain KIM5, ranging from 10 CFU to  $1 \times 10^5$  CFU. Animals that were moribund, as determined by IACUC guidelines, were euthanized; otherwise, animals were euthanized at the end of the experiment. Lungs and spleens were surgically removed and homogenized in 5.0 ml of sterile PBS by using a Stomacher Lab Blender (Seward Medical Limited), and the CFU per organ for each strain was calculated by plating serial dilutions onto TB agar.

**Histopathology and immunohistochemistry.** Mice infected *i.n.* with  $1 \times 10^5$  to  $4 \times 10^5$  CFU of *Y. pestis* strain KIM5 were euthanized at various time points postinfection (*p.i.*). All tissues were fixed in 10% buffered formalin, sectioned, dehydrated, and paraffin embedded. Glass slide-mounted serial sections were stained with hematoxylin and eosin (H&E). Following a complete necropsy of each mouse, all tissues were collected and were carefully examined grossly and microscopically. All lung lobes were blindly evaluated, and histopathological scorings were done on individual mice. For this purpose, lesion numbers were counted, and each lesion was graded as 1 (minimal), 2 (mild), 3 (moderate), or 4 (severe) by evaluating its size and the presence of edema, inflammatory cells (such as macrophages and neutrophils), and necrotic cells. A cumulative pathology score was calculated for each mouse by adding the grades of all of its pulmonary lesions. For indirect immunohistochemistry, tissue sections were treated with polyclonal rabbit anti-F1 or anti-Psa antisera that had been adsorbed with strain DSY39 (*Y. pestis psa caf*) and *E. coli* BL21(DE3) (Table 1). All reactions were performed at room temperature. Tissue sections were treated with H<sub>2</sub>O<sub>2</sub> for 10 min to inactivate endogenous peroxidases and were incubated with the primary antibody for 30 min, followed by a biotinylated anti-rabbit antibody for 15 min, horseradish peroxidase (HRP)-conjugated streptavidin for 15 min, and 3,3'-diaminobenzidine as a chromogen for 3 min. Counterstaining was done using Mayer's hematoxylin; the slides were then dehydrated, and coverslips were applied. The negative controls were prepared using N-Universal Negative Control (cocktail mixture of nonspecific rabbit IgG, IgM, IgG2a, IgG2b, and IgG3; DakoCytomation, Carpinteria, CA) instead of the primary antibodies.

**Animal immunizations and bacterial challenge studies.** For vaccination, the purified proteins were mixed with an equal volume of the adjuvant Alhydrogel or Freund's complete adjuvant (FCA) to yield a final protein concentration of 400 µg/ml. Protein-adjuvant mixtures containing 40 µg of antigen were administered *s.c.* to mice (6 to 10 mice/group), followed by one *s.c.* boost immunization of the same dose in Alhydrogel, or two *s.c.* boost immunizations of the same dose in Freund's incomplete adjuvant (FIA), at 14- to 21-day intervals post-primary vaccination. The corresponding control groups were vaccinated with Alhydrogel and 987P, Alhydrogel alone, or FCA with FIA as the boost. The vaccinated mice were challenged with *Y. pestis* KIM5 2 weeks after the last immunization.

**ELISA.** Sera collected from individual mice at the end of the vaccination trials were assayed for anti-F1 or anti-Psa IgG antibodies by a standard enzyme-linked immunosorbent assay (ELISA) on individual animals, with 0.5 µg/100 µl of either recombinant F1 or recombinant Psa applied to each well of a 96-well microtiter plate. The endpoint titers were determined by the highest serum dilutions with A<sub>450</sub> values that were at least twice the A<sub>450</sub> values for blank wells (not coated with antigen).

**Statistical analysis.** Groups of mice were compared by using the unpaired Student *t* test, and survival curves were analyzed by the Mantel-Cox log rank test (statistical significance at a *P* value of <0.05), using Prism, version 5 (GraphPad Software, Inc., La Jolla, CA). LD<sub>50</sub>s and median survival times (MST) were determined by standard methods (73).

## RESULTS

**Inorganic iron does not suppress the attenuated phenotype of *Y. pestis pgm* strain KIM5 in murine lungs.** C57BL/6 mice challenged *i.n.* with  $10^5$  CFU of *Y. pestis* KIM5 showed a lethality of only 60% with an MST of 9 days (Fig. 1A). These mice showed no noticeable macroscopic signs of pneumonia, consistent with the low numbers of bacteria in their lungs ( $\sim 10^4$  CFU) (Fig. 1B), which did not increase over time (data not shown). No significant histological changes were observed in the lungs of dead or euthanized moribund mice at days 6 to 9 (Fig. 2A and B) or in those of a mouse euthanized at an earlier time point, day 3 (not shown). In contrast, the spleens of these mice had moderate hyperplasia and sporadic single-lymphocyte death (not shown) with significantly higher numbers of strain KIM5 than numbers in lungs (Fig. 1B), revealing bacteremic spread. These results indicated that *pgm* strain KIM5 administered into the respiratory tract kills mice following bacteremia without the typical pneumonic lesions and the concomitant uncontrolled bacterial growth characteristic of pneumonic plague induced by fully virulent strains (32). *Y. pestis pgm* mutants lack the yersiniabactin-dependent iron transport system, which was shown to be essential for plague infection from *s.c.* or *i.p.* injections (52). Since earlier studies had shown that the need for yersiniabactin can be bypassed by iron treatment in murine models of bubonic plague (10), we undertook experiments to determine whether *i.n.* infection of iron-treated mice with a *Y. pestis pgm* mutant can recapitulate the bacterial multiplication and lung lesions typical of pneumonic plague. Iron sulfate administered *i.p.* (0.4 mg for the first day and 0.2 mg per following day) to C57BL/6 mice before an *i.n.* challenge with  $10^5$  CFU of strain KIM5 did not result in bacterial growth accompanied by pulmonary lesions (data not shown). Lee-Lewis and Anderson recently described comparable results in a study with BALB/c mice challenged *i.n.* with *pgm* strain KIM D27, where despite persistent low-level colonization of the lungs, the typical pulmonary inflammation and disease pathology of pneumonic plague were absent (33).

**Iron dextran suppresses the attenuated phenotype of *Y. pestis pgm* strain KIM5 in murine lungs.** Iron is rapidly eliminated or rendered unavailable in hosts injected with inorganic iron, and the profound toxicity of inorganic ferric iron unmixed with carbohydrates precludes its administration at high doses (16). Thus, we decided to test iron dextran, which is significantly less toxic and is available for a longer period in the circulation (16, 34). When the *i.n.* challenge with strain KIM5 ( $1 \times 10^5$  CFU) was accompanied by daily administration of 10 mg iron dextran *i.p.* together with one *i.n.* delivery of 2 mg iron dextran 2 to 3 h postinfection, general disease symptoms such as weight loss

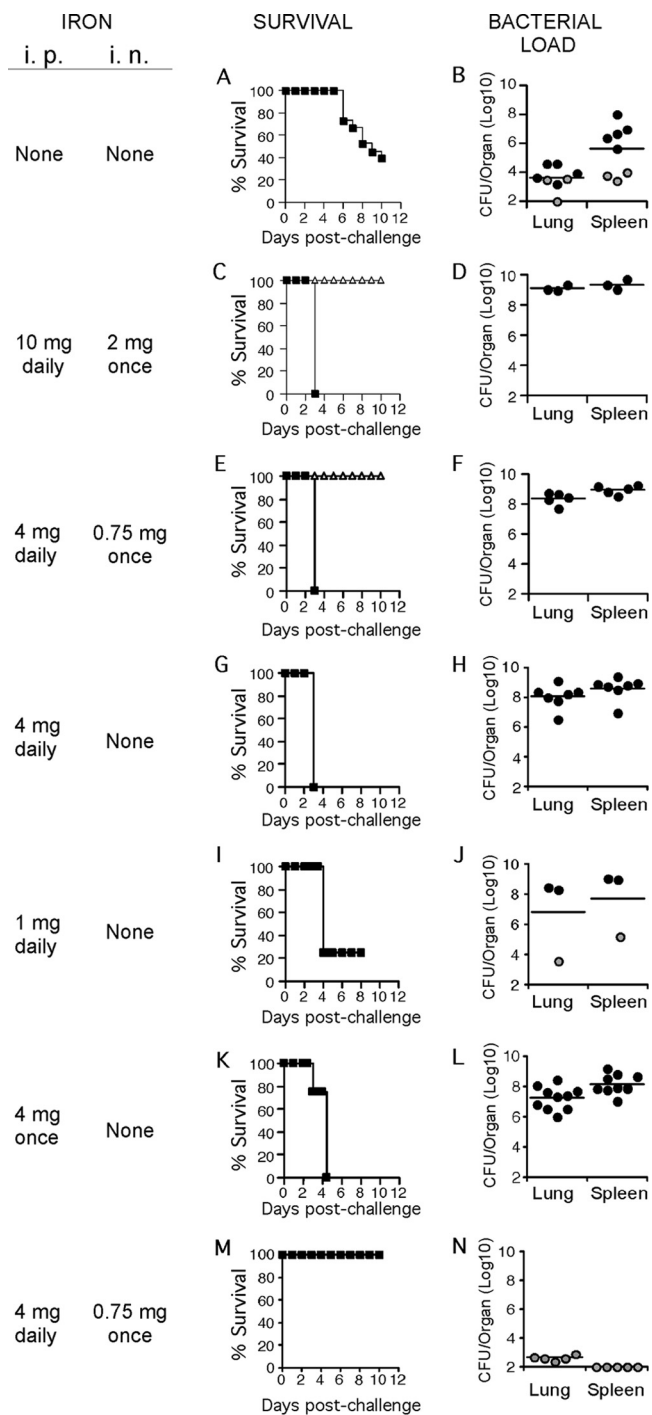


FIG. 1. Survival and bacterial loads in organs of iron-treated mice infected i.n. with *Y. pestis*. Groups of mice underwent different regimens of iron supplementation by receiving iron dextran i.n. and/or i.p. as indicated. Mice were challenged i.n. with  $1 \times 10^5$  CFU *Y. pestis* strain KIM5 (A to L) or KIM6 (M and N). Survival was assessed by monitoring the mice twice daily for 10 days (A, C, E, G, I, K, and M). Bacterial loads in the lungs and spleen (B, D, F, H, J, L, and N) were quantified by CFU counts from tissue homogenates (euthanized mice with terminal symptoms) (filled circles) or at the end of the experiment with surviving mice (open triangles) in panels C and E. The solid lines represent corresponding geometric means. Data were from three independent experiments, each of them with 5 mice (A and B); from one experiment with 5 mice per group (C and D, E and F, I and J, M

and lethargy typically appeared in 2 days, followed by a 100% lethal outcome at 72 h (Fig. 1C), with high bacterial counts in both lungs and spleens (Fig. 1D). At the time of death or just before death, in contrast to the lungs of infected but untreated mice (Fig. 2A and B), the lungs of iron dextran-treated mice showed large focal aggregates of bacteria with a multifocal neutrophilic and histiocytic infiltrate effacing the alveolar network (Fig. 2C and D). A set of further experiments was undertaken to determine whether lower doses of iron dextran were still able to induce pneumonia in mice infected i.n. with strain KIM5. Comparable lethal effects (Fig. 1C, E, and G) and essentially corresponding high bacterial counts were obtained with daily administration of 10 mg or 4 mg iron dextran by the i.p. route, with or without additional i.n. administrations of iron dextran 2 to 3 h before infection (Fig. 1D, F, and H). In mice receiving 4 mg iron dextran daily, lungs were affected by multifocal inflammatory foci that were characterized by complete effacement of the parenchyma via numerous neutrophils and necrosuppurative lesions with karyorrhexis (Fig. 3A and B). Spleens were affected by red pulp necrosis with lymphocyte loss and mesothelial hypertrophy (not shown). When the amount of iron dextran was decreased to a daily i.p. application of 1 mg, bacterial counts in the lungs and spleens of affected mice (Fig. 1J) were almost as high as those observed when larger iron doses were administered. However, the fatality rates decreased, dropping to 75% (Fig. 1I). In contrast, a single injection of 4 mg iron dextran i.p. given on the same day as the bacterial challenge caused 100% death after 4.5 days (Fig. 1K). Moreover, the bacterial counts in the lungs and spleens were well over 1,000 times higher than those found in the organs of mice that received no iron, reaching  $10^7$  to  $10^8$  CFU per organ (Fig. 1L). Histology of the lungs delineated clear inflammatory foci composed mainly of neutrophils and proteinaceous fluid (Fig. 3C and D), contrasting with the total absence of such lesions in mice administered only iron dextran (Fig. 3E and F). To quantitatively evaluate how the different iron dextran treatments affected the pulmonary histopathology of the infected mice, pathological scores reflecting the numbers and extents of focal inflammatory lesions were recorded as described in Materials and Methods. Infected mice given 4 mg iron dextran daily showed scores significantly different from those of mice given the same treatment only once ( $P < 0.019$ ), and infected mice treated only once with iron dextran showed significantly higher scores than infected but untreated mice ( $P < 0.034$ ) (Fig. 3G). This result indicated that the severity of pneumonia was modulated by the iron dextran dosage; a single or a daily dose of 4 mg led to light or severe pneumonia, respectively. As an additional negative control, iron dextran-treated mice (4 mg i.p. daily and 0.75 mg once i.n.) were challenged i.n. with strain KIM6, which lacks the virulence plasmid pCD1. All the mice

and N); from two independent experiments, each of them with 5 mice (G and H); or from one experiment with 10 mice (K and L). The survival curve in panel A was significantly different from those in panels C, E, G, or K ( $P < 0.001$  by the log rank test). Bacterial loads in the lungs were significantly different from those in the spleen in panel B ( $P < 0.005$ ). Bacterial loads in both the lungs and the spleen were significantly different for panel B versus panel L ( $P < 0.001$ ).

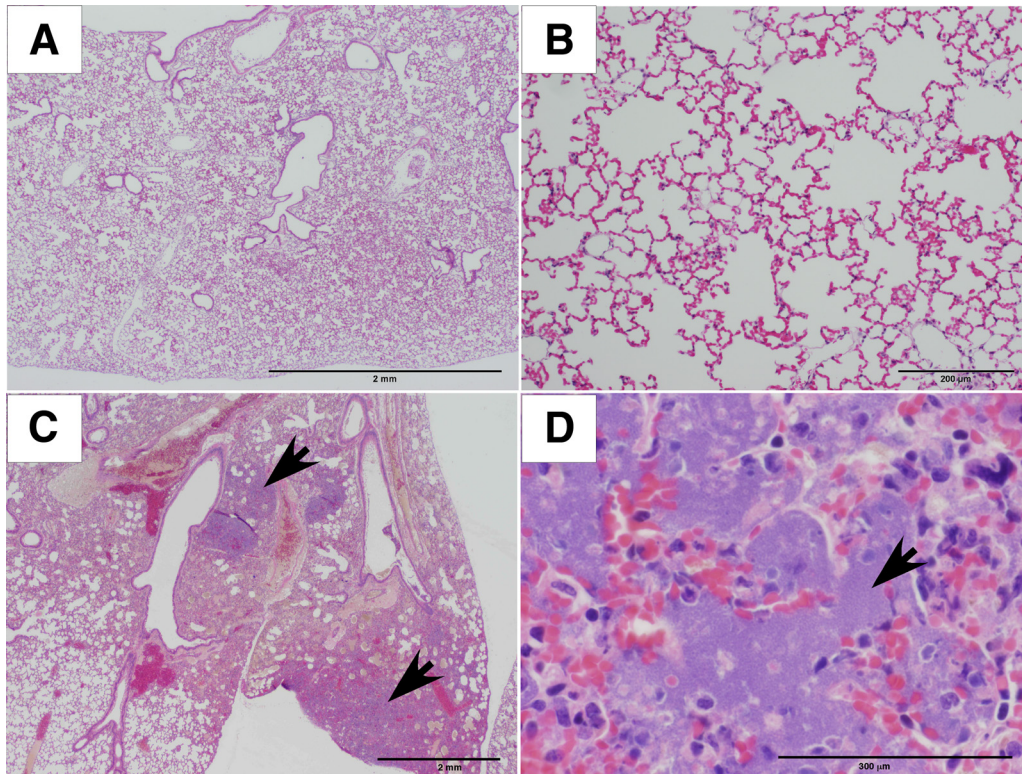


FIG. 2. Lung histopathology of mice infected i.n. with *Y. pestis* strain KIM5. Mice received either no iron dextran (A and B) or 10 mg iron dextran i.p. 2 to 3 h before infection and daily thereafter, with an additional dose of 2 mg i.n. 3 h p.i. (C and D). Mice were challenged with  $10^5$  CFU of strain KIM5. (A and B) Representative lungs of mice that died or were euthanized moribund at day 6 to 9 (see Fig. 1A) or that were euthanized after 10 symptomless days p.i. (C and D) Representative lungs of mice that succumbed to the infection 2.5 days p.i. were fixed with 0.4% formaldehyde, and sections were stained with H&E. Arrows indicate masses of bacteria. Bars, 2 mm (A and C), 200  $\mu$ m (B), and 300  $\mu$ m (D).

survived (Fig. 1M), and the CFU counts were negligible 10 days after infection (Fig. 1N), confirming that iron dextran by itself had no significant deleterious effect on mice and that the iron dextran-promoted virulence detected was specific for *pgm* strain KIM5.

Taken together, our data showed that the pathology of pneumonic plague could be recapitulated with strain KIM5 by pretreating the mice with iron dextran. Most importantly, the lesions observed compared well with those described previously for pneumonic plague induced by wild-type strains (3, 25, 32). Since a higher dose of iron dextran increased the severity of the infectious process, it was not surprising that it also decreased the  $LD_{50}$ . With the least aggressive iron dextran treatment that still induced distinctively pneumonic plague, namely, 4 mg i.p. once, the  $LD_{50}$  of KIM5 was determined to be 760 CFU. In contrast, the  $LD_{50}$  of KIM5 was determined to be 31 CFU with the daily administrations of 4 mg iron dextran i.p., which induced more-extensive pneumonic lesions. These values compared well with literature data showing an  $LD_{50}$  of 330 CFU for strain KIM given i.n. to BALB/c mice (19, 69), or 40 to 1200 CFU for strain CO92 administered i.n. to C57BL/6 mice (9, 30, 32).

***In vivo* expression and antigenicity of F1 and Psa.** Having shown that strain KIM5 can induce primary pneumonia in iron dextran-treated C57BL/6 mice, we determined whether this infection model would be useful for protection studies with

experimental vaccines. The currently best studied experimental vaccine against pneumonic plague consists of the F1 and LcrV antigens, each of which by itself has protective properties against virulent wild-type strains of *Y. pestis*, such as strains KIM and CO92 (60). Thus, to further validate the pneumonic plague model with iron dextran-treated mice and strain KIM5, it was essential to demonstrate F1 antigen expression in this model. The immunogenicity and protective properties of F1 can be attributed to its efficient *in vivo* expression as a homopolymeric surface protein. Since, like F1, the Psa fimbria of *Y. pestis* is also exported as a homopolymer by a chaperone-usher apparatus, Psa fimbrial protein was investigated in parallel with the F1 antigen. Strain KIM5 was shown by immunohistochemistry to express both the F1 and Psa antigens in the spleens of infected mice, irrespective of iron dextran administration (Fig. 4A, B, D, E, and G [negative control]). The two antigens were also detected in the lungs and bronchial lymph nodes, though only for iron dextran-treated mice (Fig. 4C, F, H, and I); bacterial numbers were probably too low for visualization in the lungs of untreated mice (not shown). Bacterial expression of F1 and Psa in the lungs was independent of the iron dextran dosage studied (4 mg once or daily). Having demonstrated *in vivo* expression of F1 and Psa, we next aimed to investigate the immunogenicity of these two antigens. For this purpose, C57BL/6 mice ( $n = 5$ ) were immunized with two doses of 40  $\mu$ g recombinant antigen in Alhydrogel. Since our

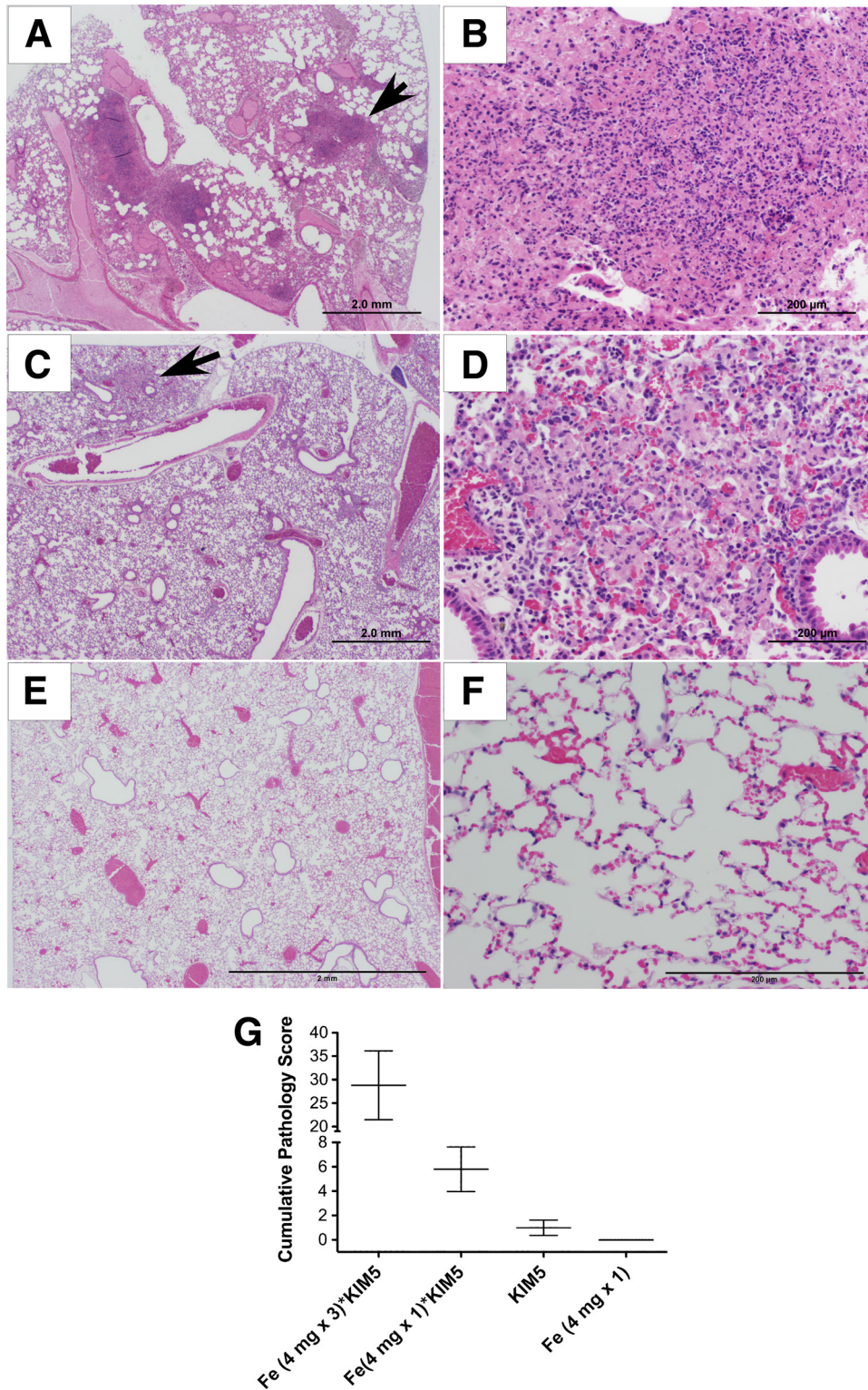


FIG. 3. Lung histopathology of iron dextran-treated mice infected i.n. with *Y. pestis* strain KIM5. Mice received 4 mg iron dextran i.p. either daily (A and B) or once (C, D, E, and F) and either were infected i.n. with  $10^5$  CFU of strain KIM5 (A to D) or received PBS i.n. (negative control) (E and F). Lungs of mice that either succumbed to the infection 2.5 to 4 days p.i. (A to D) or were euthanized 9 days p.i. (E and F) were fixed with 0.4% formaldehyde, and sections were stained with H&E. Arrows in the left panels indicate the areas shown on the right panels for the same row. Bars, 2 mm (A, C and E) or 200  $\mu$ m (B, D, and F). None of the iron dextran-treated uninfected mice showed any lesions (E and F). (G) Lung pathology scores showed significant differences between the infected mice that were treated daily and those treated once with iron dextran ( $P < 0.019$ ), and the infected mice treated only once with iron dextran showed significantly higher scores than the infected but untreated mice (see Fig. 2A and B) ( $P < 0.034$ ).

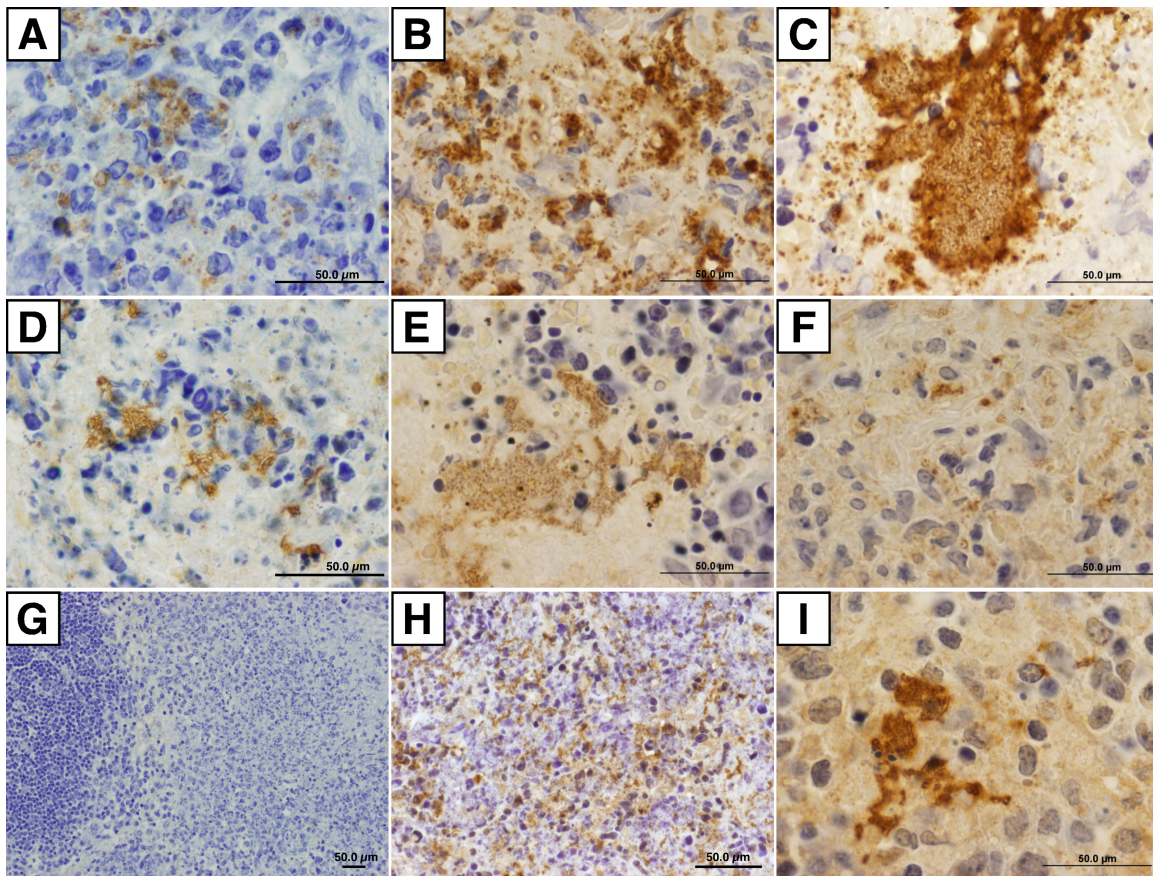


FIG. 4. *Y. pestis* expression of the F1 antigen or Psa in infected mice. Immunohistochemistry of organs from mice challenged i.n. with  $10^5$  *Y. pestis* KIM5 bacteria was performed. Murine tissues were stained with hematoxylin (blue). Bacteria expressing the F1 antigen (in brown) were detected with specific antisera in the spleens of mice that received no iron (A) or 4 mg iron once (B), as well as in the lungs of the latter mice (C). Bacterial numbers in the lungs of the former mice were too low for detection (see Fig. 1B). Similarly, bacteria expressing Psa were detected in the spleens of mice that received no iron (D) or 4 mg iron once (E), as well as in the lungs (F) and bronchial lymph nodes (H) of the latter mice, but not in the lungs of the former mice (not shown). Bacteria expressing Psa were also detected in the lungs of mice treated daily with 4 mg iron dextran (I). No bacteria were visualized in tissues stained with nonspecific antibodies (G). Organs were from day 2.5 (I), 4 (B, C, E, F, and H), or 5 (A, D, and G) p.i. Bars, 50  $\mu$ m.

recombinant *E. coli* expressing Psa from plasmid pCS267 (37) released large amounts of the Psa antigen into the medium, we isolated and tested both released and bacteria-associated antigens. Although the latter Psa antigen gave slightly better titers (one 2-fold dilution), each antigen (F1 and the two Psa protein preparations) induced a strong humoral immune response (geometric mean IgG titer,  $>10^5$ , as determined by ELISA 2 weeks after the last immunization). Taken together, the data showed that F1 and Psa are expressed *in vivo*, particularly in affected lungs of iron dextran-treated mice, and that the Psa protein is as immunogenic as the F1 antigen. In addition the results were consistent with human data, which strongly suggested that the Psa protein induces strong immune responses, since the invading *Y. pestis* elicited anti-Psa antibodies in plague patients (35).

**Iron dextran-treated KIM5-infected mice: a pneumonic plague model for antigen protection assays.** Having shown that an iron dextran-treated mouse model could be used to mimic pneumonic plague with strain KIM5, we determined whether the known protective antigen F1 demonstrates protection with this model. Psa was also investigated with this model, since it was

expressed *in vivo*, was a strong immunogen, and appeared to have protective properties in a preliminary study. The latter study had tested the protective properties of Psa with mice that were not treated with iron. Three groups of 10 C57BL/6 mice had been immunized s.c. with the F1 antigen, Psa, or the *E. coli* 987P fimbriae as a negative control (40  $\mu$ g of purified antigen injected on days 0 and 21). On day 35 postimmunization, non-iron-treated mice were challenged i.n. with  $6.6 \times 10^6$  CFU of strain KIM5. As expected, immunization with F1 provided 100% protection. Interestingly, all the Psa-immunized mice were also protected, while 60% of control mice immunized with the unrelated fimbrial protein 987P died ( $P$ , 0.0042 for comparison of the F1 or Psa against the 987P data). Consistent with these observations, Psa- and F1-immunized mice did not show any significant decrease in weight, whereas all the mice immunized with 987P fimbriae lost weight ( $P$ ,  $<0.001$  for comparison of the F1 or Psa against the 987P data), and those that survived began to recover at day 7 postinfection (data not shown). Although the data for the control mice indicated that the challenge dose was barely above 1 LD<sub>50</sub>, the result for the Psa antigen warranted its further inclusion in protection ex-

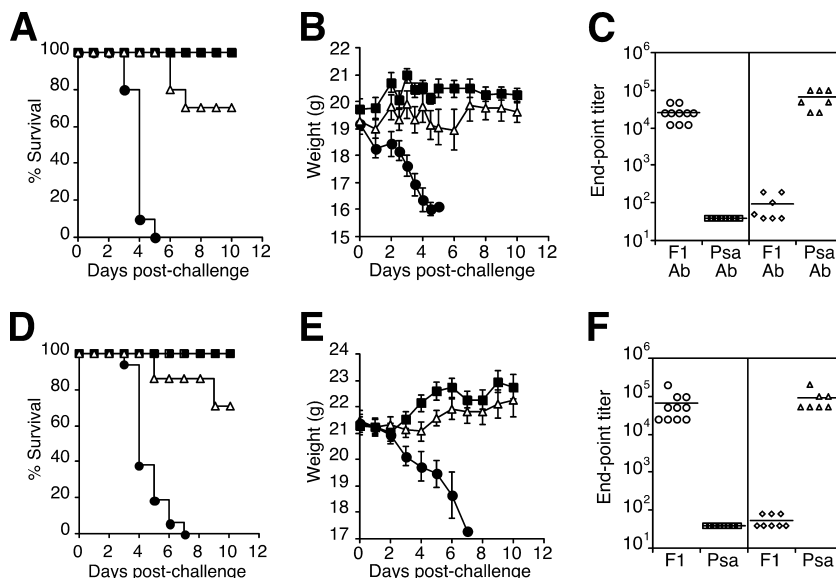


FIG. 5. Challenge of F1 antigen- or Psa-immunized mice. Groups of 7 to 10 mice were immunized s.c. with Alhydrogel (A, B, and C) or Freund's adjuvant (D, E, and F) either alone (filled circles) or together with the F1 antigen (filled squares) or Psa (open triangles). Mice received either one boost immunization at a 3-week interval (A, B, and C) or two boost immunizations at 3- and 2-week intervals (D, E, and F). Two weeks following the last boost immunization, mice were administered 4 mg iron dextran i.p. 2 to 3 h before challenge with 210 LD<sub>50</sub>s (A, B, and C) or 17 LD<sub>50</sub>s (D, E, and F) of *Y. pestis* KIM5 by i.n. instillation. The second group of mice received additional administrations of 4 mg iron dextran i.p. at days 2 and 3. Survival (A and D) and weight (B and E) were monitored for 10 days. F1-specific and Psa-specific antibodies were measured by ELISAs in sera from F1-immunized mice (C and F, left panels), and from Psa-immunized mice (C and F, right panels).

periments with iron dextran-treated mice. For this purpose, an experiment was carried out with C57BL/6 mice ( $n = 10$ ) immunized with two doses of 40  $\mu$ g of recombinant F1 antigen or Psa isolated from bacterial surfaces, mixed with the adjuvant Alhydrogel. Two challenge doses were studied by infecting iron dextran-treated mice (one dose of 4.0 mg i.p.) i.n. with 210 or 21 LD<sub>50</sub>s of *Y. pestis* KIM5. As expected, all the control mice that were immunized with the adjuvant alone died 3 to 5 days after immunization (MST, 4 days) with 210 LD<sub>50</sub>s (Fig. 5A). In contrast, 100% of the F1 antigen-immunized mice and 70% of the Psa-immunized mice survived the bacterial challenge with 210 LD<sub>50</sub>s ( $P < 0.0001$  for comparison of the F1 or Psa against the adjuvant-alone data;  $P > 0.05$  for comparison of the F1 against the Psa data), and all the deaths in the Psa-immunized group occurred between days 6 and 7. No significant weight variations were observed for the F1- or the Psa-immunized mice that survived (Fig. 5B). All the control mice gradually lost weight after the second day postinfection, whereas the Psa-immunized mice that became sick began to lose weight only after day 3 to 4 postinfection. In these experiments, the F1 antigen, as well as Psa, induced high immunogen-specific antibody titers in mice, with endpoint titers of 10,000 to 100,000 (Fig. 5C). Comparable results were obtained for mice infected with 21 LD<sub>50</sub>s, with 100% of F1-immunized mice and 80% of Psa-immunized mice surviving the challenge ( $P < 0.002$ ) (data not shown).

To test the iron dextran-treated mouse model more stringently, additional experiments were undertaken with F1 antigen- or Psa-immunized mice by administering the iron dextran regimen (4 mg daily) shown to induce severe and extensive pneumonia in KIM5-infected mice. Moreover, since Psa did not protect as well as the F1 antigen in the earlier experiment,

and in order to evaluate the potential protective properties of Psa with a more powerful adjuvant, the new experiments were undertaken with Freund's adjuvant. Ten C57BL/6 mice were immunized with 40  $\mu$ g antigen in FCA, with two boosts of the same dose in FIA, at 2- to 3-week intervals. In a first experiment, the immunized and iron dextran-treated mice were challenged i.n. with 17 LD<sub>50</sub>s of *Y. pestis* KIM5. All the control mice immunized with the adjuvant alone died 3 to 7 days p.i. (MST, 4 days) (Fig. 5D). In contrast, 100% of the F1 antigen-immunized mice and 70% of the Psa-immunized mice survived this bacterial challenge ( $P < 0.0001$  for comparison of the F1 or Psa data against the adjuvant-alone data;  $P > 0.05$  for comparison of the F1 against the Psa data). In the Psa-vaccinated group, all the deaths occurred between days 5 and 9. No significant weight variations were observed for the F1 antigen-immunized mice (Fig. 5E). All the control mice gradually lost weight after the second day p. i., whereas the Psa-immunized mice started to gain weight by day 5 after an initial weight loss on days 3 and 4 p.i. In these experiments, the F1 antigen as well as Psa induced high immunogen-specific antibody titers in mice, with endpoint titers of 10,000 to 100,000 (Fig. 5F). In contrast to the significant but partial protection of the Psa-immunized mice in this experiment, Psa was not protective when the bacterial challenge dose was increased to 170 LD<sub>50</sub>s. Although all the F1 antigen-immunized mice survived, all the Psa-immunized mice died, as did the control (adjuvant-alone) mice. However, the survival data were significantly different for the latter two groups ( $P, 0.028$ ), with survival times of 4 to 9 (MST, 5 days) and 3 to 6 (MST, 3 days) days p.i. for the Psa-immunized mice and the control mice, respectively. In summary, these results indicated that the F1 antigen is similarly protective against pneumonic plague whether a wild-type



strain is used to challenge mice, as described in the literature (4, 58, 60), or whether iron dextran-treated mice are challenged with strain KIM5. Moreover, the data suggested that Psa has some protective properties for mice affected by pneumonic plague, although they are less impressive than those of the F1 antigen, since significant protective effects against severe pneumonia were detected only when the i.n. bacterial challenge was not in excess of 17 LD<sub>50</sub>s.

## DISCUSSION

Various experimental animals have been used to model human pneumonic plague. Most studies in the literature have focused on the murine model, particularly the more recent investigations, which have begun to unravel the contributions of cellular immune components to the benefit of vaccine development (60, 61). Although early live vaccines and several promising new mutants have been shown to deliver protective properties, they still have poor or unknown safety records (1, 38, 41, 46, 47, 63, 65). By definition, an ideal vaccine should be not only 100% protective but also 100% safe. Thus, a killed vaccine or a multisubunit vaccine that affords full protection is the most desirable vaccine. Because killed vaccines have proven insufficient in providing protection against pneumonic plague (41, 60), most research to date has focused on the experimental F1 and LcrV subunit vaccine, which protects mice against bubonic and pneumonic plague (58). Although protection proved insufficient in an analogous primate model (1, 55, 60), this vaccine appears to be safe and immunogenic in humans (74). However, the ability of F1-deficient strains of *Y. pestis* to cause pneumonic plague in mice and primates, and the consequent reliance on just one immunogen for protection, highlights the need to identify additional protective subunits (1, 40, 45, 60). A variety of protein subunits, such as the YpkA (5), YopD (5), YscC (24), YscF (40, 64), and YadC (45) proteins of *Y. pestis*, have been shown to possess some protective properties. Unfortunately, the experimental protocols used to detect protection are so varied that a comparative evaluation of the different immunogens is not possible. A major hurdle for the identification and characterization of new protective immunogens is the need to undertake challenge experiments with wild-type strains of *Y. pestis* and thus to use BSL-3 conditions. Here we tested the possibility of using strain KIM5 in a new BSL-2 model of pneumonic plague. Strain KIM5 lacks the *pgm* locus, which encodes, among others, an essential virulence factor for nonintravenous plague infections, namely, the yersiniabactin-dependent iron transport system (52). Disease progression induced by *Y. pestis pgm* strains, such as strain KIM5, does not recapitulate that observed with fully virulent strains (19, 33, 44). As recently reported by others, we were unable to infect mice successfully by administering KIM5 by the i.n. route; the bacteria multiplied poorly in the lungs, which showed no significant histopathology. Early studies indicated that iron injections given to mice could suppress *pgm* attenuation in *Y. pestis* (10). However, a detailed recent study based on the administration of iron chloride to mice demonstrated the absence of pneumonic lesions after i.n. challenge with strain KIM D27 (33). Although we observed similar results with iron sulfate, we were able to recapitulate pneumonic plague following i.n. infection with strain KIM5 in iron dex-

tran-treated mice. Survival curves and strain KIM5 multiplication in the lungs were similar to those described elsewhere for wild-type strains (3, 32). Moreover, the F1 antigen was clearly detectable in the lungs and spleens of KIM5-infected mice.

Interestingly, Fetherston et al. have recently shown that the i.n. LD<sub>50</sub> of *Y. pestis irp2* (*irp2* being a yersiniabactin biosynthetic gene) was ~670 times higher than that of the wild-type strain, whereas the i.n. LD<sub>50</sub> of *Y. pestis pgm* was ~12,000 times higher than that of the wild-type strain (19). This >15-fold-increased lethality highlighted the presence of at least one other virulence factor in the *pgm* locus; the yersiniabactin locus occupies only a portion of it. An obvious candidate virulence factor could be the *ripA* gene (57), although Bliska and Perry reported that a  $\Delta ripABC$  mutant was fully virulent in mice (cited in reference 19). Even though several other genes of the *pgm* locus have been proposed to be involved in this increased virulence of pneumonic plague, the effect of such genes on LD<sub>50</sub>s was significantly less impressive than the effect of *irp2* (19). Thus, the yersiniabactin locus has to be considered the major player in the virulence attributed to the *pgm* locus, in support of our use of iron dextran to bypass *pgm* attenuation. Moreover, in contrast to the i.n. LD<sub>50</sub> of strain KIM5 (>3.9 × 10<sup>6</sup> CFU), the LD<sub>50</sub>s of strain KIM5 with the iron dextran-treated mouse model of pneumonic plague (30 to 760 CFU) and the parental wild-type strain KIM with untreated mice (330 CFU) were essentially in the same range (19, 69), strongly suggesting that the predicted additional *pgm* virulence factor did not significantly influence the parameters for pneumonic plague tested here. Nevertheless, a potential caveat for iron-treated mouse models is that iron can influence the host's innate immune responses, for example, by allowing virulent bacteria to multiply better in iron-rich phagocytes (22, 42, 70). Also, administration of iron renders mice more susceptible to human pathogens, a method that has been used to develop a variety of infectious-disease models (62, 71, 75). Thus, the KIM5-iron dextran model of pneumonic plague might be considered too stringent if it were used to study the protective properties of potential vaccines or the effects of attenuation with mutants of potential virulence factors. For this reason, the model was further tested in protection assays with the F1 immunogen, a *Y. pestis* immunogen known to protect against i.n. infections with wild-type strains. F1-immunized mice were fully protected in our model, consistent with the protective property of this antigen for murine pneumonic plague (4) and further supporting the use of the iron dextran-treated mouse model of primary pneumonic plague with strain KIM5. This simple, safe, and cost-reducing BSL-2 model of infection should be valuable for exploratory investigations aimed at identifying the few bacterial antigens or virulence factors that should be further characterized in infection models with fully virulent wild-type strains that require BSL-3 facilities.

*Y. pestis* Psa fimbriae were previously found by us and others to bind to respiratory tract epithelial cells and interact with macrophages (31, 37). Although the results of previous studies indirectly suggested that the Psa fimbriae are expressed *in vivo* (7, 13, 14, 35, 36), definitive evidence was lacking. In this study we have conclusively demonstrated by immunohistochemistry that the Psa antigen is produced in tissues of *Y. pestis*-infected mice. Psa harbors a major desirable property of a protective immunogen for vaccine design in that it is conserved in all

currently sequenced *Y. pestis* strains. We showed here that, consistent with human data (35), Psa is immunogenic in mice and has significant protective properties in the iron dextran-treated mouse model of pneumonic plague, although they are less dramatic than those of F1. Our results contrasted with a reference to unpublished data (cited in reference 66) and a recent study that did not detect Psa-mediated protection (7). However, a different antigen preparation technique, a lower antigen dose, the absence of an adjuvant, and challenge with a different bacterial strain by another route (s.c.) in the latter study could each explain this discrepancy. Although the results of the current study will have to be confirmed by wild-type strain infection challenges, they provide a sound basis for further evaluation of Psa as a vaccine component.

This study and other studies have now identified several *Y. pestis* proteins (61) that might serve to complement the current experimental acellular *Y. pestis* vaccine based on the F1 and LcrV immunogens. A multicomponent vaccine against pneumonic plague could be modeled on the successful acellular *Bordetella pertussis* cocktail vaccines, all of which include several proteins, and those with three or more components are considered to be more effective than one- or two-component vaccines that contain only pertussis toxin (PT) or both PT and filamentous hemagglutinin (FHA) (27, 56). Thus, it is expected that a cocktail of *Y. pestis* immunogens will not only elicit an immune response capable of blocking bacterium-host interactions but will also broaden the range of protected individuals. Moreover, a vaccine consisting of multiple *Y. pestis* immunogens will have the advantage of extending protection against natural mutants and genetically engineered mutants intentionally designed to remain pathogenic.

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