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Fraction 1 Capsular Antigen (F1) Purification from *Yersinia pestis* CO92 and from an *Escherichia coli* Recombinant Strain and Efficacy against Lethal Plague Challenge

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As a first step in formulating an improved plague vaccine, we developed a simple purification strategy that produced high yields of pure cell-associated and culture supernatant-derived fraction 1 capsular antigen (F1) from both avirulent *Yersinia pestis* CO92 (Pgm^- Lcr^-) and an *Escherichia coli* F1-producing recombinant strain. Cell-associated F1 was partially purified by sequential ammonium sulfate precipitations of a sodium chloride extract of acetone-dried bacteria harvested from broth cultures. Cell-free F1 was precipitated directly from culture supernatants with a single application of 30% ammonium sulfate. By exploiting the aggregative property of F1, large quantities of purified high-molecular-weight F1 species from both cell extracts and supernatants were isolated in the void volume of a preparative gel filtration column. Highly purified, endotoxin-free F1, combined with two different adjuvants, induced very high F1 titers in mice and protected them against either subcutaneous (70 to 100% survival) or aerosol (65 to 84% survival) challenge with virulent organisms. This protection was independent of the source of the antigen and the adjuvant used. F1-induced protection against both subcutaneous and aerosol challenge was also significantly better than that conferred by immunization with the licensed killed whole-cell vaccine. Our results indicate that F1 antigen represents a major protective component of previously studied crude capsule preparations, and immunity to F1 antigen provides a primary means for the host to overcome plague infection by either the subcutaneous or respiratory route.

Yersinia pestis fraction 1 capsular antigen (F1) is a plasmid (pFra)-encoded, proteinaceous capsule, synthesized in large quantities by the pathogen, and reported to confer antiphagocytic properties on *Y. pestis* by interfering with complement-mediated opsonization (40). The protein is highly immunogenic and has been indirectly associated with eliciting a protective immune response in humans, as evidenced by the detection of high levels of anti-F1 antibody in F1-immunized volunteers (28). Hyperimmune sera from these individuals possessed F1 antibody levels comparable to those levels found in human convalescent sera and were subsequently shown to passively protect mice from virulent plague challenge (28). Direct evidence that F1 can function as a protective immunogen was originally reported by Baker and others (4) and confirmed by Zakrevskiy and Plekhanova (41). Recently, as little as 1 μg of recombinant F1 from an acrylamide gel cut protected mice against 10^5 parenterally administered virulent organisms (33). Furthermore, mice immunized with live recombinant *Salmonella* strains expressing F1 were protected against a parenteral plague challenge (16, 29). Although few experimental data exist for protection elicited by F1 against aerosolized *Y. pestis*, early studies with nonhuman primates suggest that hyperimmunization with the antigen can partially protect against the pathogen (11).

The current licensed vaccine for plague consists of killed whole cells of virulent *Y. pestis* (27). Efficacy conferred by the vaccine against bubonic plague has been suggested by epidemiological studies in humans and directly demonstrated in experimental animals (8, 27, 39). Despite this finding, the primary protective components in this preparation remain undefined, although F1 may, at least, partially contribute to the protection conferred by the vaccine (39). Additionally, there is no evidence demonstrating protective efficacy of the licensed vaccine against pneumonic plague.

The structural gene for F1 is temperature regulated (10, 33) and is part of an operon (*caf*) which also encodes a *trans* activator (21), a periplasmic binding protein (14), and a putative anchor protein (22), which are all required for full expression and secretion of F1 (33). The *caf* operon has been cloned into *Escherichia coli* (33), and the structural gene and the accessory loci have been sequenced (14, 15, 21, 22). The size of the antigen subunit, predicted by the coding sequence of the structural gene, agreed well with previous studies which reported a 17.0- to 17.6-kDa polypeptide with an isoelectric point of 4.1 to 4.4 in denaturing polyacrylamide and isoelectric focusing (IEF) gels (32, 37). The F1 open reading frame also predicts a hydrophobic protein with a β-sheet secondary structure (15), which correlates with the observation that the subunit forms aggregate under physiological conditions (5, 38). Additionally, an early in vitro study suggested that the F1 capsule is released from the cells (12).

Baker and coworkers (4) first extracted F1 with a solution of toluene-saturated sodium chloride from acetone-dried *Y. pestis* grown on agar plates. The antigen was partially purified by differential ammonium sulfate precipitations, which yielded two F1-containing fractions, 1A and 1B. Fraction 1A consists of protein and carbohydrate, whereas fraction 1B is composed entirely of protein. Although F1 antigen was the primary component of both fractions, other *Y. pestis*-specific antigens, including lipopolysaccharide (LPS), most likely were present in these preparations, which had been used for early plague protection studies in an animal model (4). Therefore, to determine the contribution of F1 to the protective immune response

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against plague, we extended the Baker extraction technique and developed a scheme that produced highly purified, endotoxin-free preparations of F1 from *E. coli* F1-producing recombinant strains as well as an avirulent F1-producing strain of *Y. pestis*. We then examined cell-associated F1 as well as cell-free culture supernatant F1 from both sources for their protective efficacy against subcutaneous (s.c.) and aerosol wild-type *Y. pestis* challenge.

MATERIALS AND METHODS

Bacterial strains and reagents. Wild-type *Y. pestis* CO92 was obtained from the laboratory of T. Quan, Centers for Disease Control, Fort Collins, Colo. A Pgm^- derivative of this strain was obtained by serial passage on Congo red medium (35) and was cured of the 75-kb low-calcium response (Lcr) virulence plasmid by selection at 37°C on magnesium oxalate agar (20). The variant was completely avirulent in mice at 2×10^6 to 3×10^6 organisms by the parenteral route and was subsequently used for all *Y. pestis*-derived F1 preparations. *E. coli* (pYPR1), which expressed F1 from the cloned *caf* operon, was obtained from T. Schwan, Rocky Mountain Laboratories, Hamilton, Mont. A commercial killed whole-cell plague vaccine preparation (lot no. 10K02A; Miles, Inc., Cutter Biological, Elkhart, Ind.) was used as the positive control for virulent challenge experiments.

Wild-type *Y. pestis* CO92, used for all challenges, was grown for 24 h at 30°C in heart infusion broth (HIB; Difco Laboratories, Detroit, Mich.) supplemented with 0.2% xylose. The broth cultures were then centrifuged, washed with fresh HIB, adjusted to an A_{620} of 10.0, and diluted to the appropriate challenge dose.

Adjuvants used in combination with purified F1 preparations were aluminum hydroxide (Alhydrogel; Superfos Biosector, Vedbaek, Denmark) and R-730 emulsion (Ribi ImmunoChem Research, Inc., Hamilton, Mont.) which contained 50 μg of monophosphoryl lipid A, 50 μg of synthetic trehalose dicorynomycolate, and 50 μg of cell wall skeleton in 2.2% squalene and Tween 80 per 0.2-ml vaccine dose.

An F1-specific monoclonal antibody used to detect capsular antigen was obtained directly from culture supernatants of hybridoma fusion F1-3G8-1 (HB 192; American Type Culture Collection, Rockville, Md.).

Genetic manipulations. To stabilize the cloned *caf* operon for batch production of F1 from an *E. coli* recombinant strain, standard genetic manipulations (31) were used to transfer the original 9.4-kb *Eco*RI fragment, containing the F1 coding sequences (33), from pBluescript (Stratagene, La Jolla, Calif.) to the medium-copy-number vector pBR322 (7). Transformants of *E. coli* HB101 containing the new recombinant plasmid, designated *E. coli*(pBRF1), were selected on L agar supplemented with 100 μg of ampicillin per ml. Overnight broth cultures of selected transformants were tested for the ability to stably express F1 capsule by staining the organisms with India ink (3). Individual cells possessing a capsule were identified by the ability to display a light-refractory halo under phase-contrast microscopy.

Preparation of crude F1. Partially pure cell-associated (capsular) F1 was extracted and isolated by a modification of the method of Baker et al. (4). Ten milliliters of HIB containing 0.2% xylose was inoculated with a loop of *Y. pestis* CO92 Pgm^- Lcr^- from a plate stock and grown for 24 h at 37°C. Two to four liters of HIB were subsequently inoculated with the seed cultures and grown for 48 to 72 h at 37°C with agitation at 175 rpm. *E. coli*(pYPR1) and *E. coli*(pBRF1) were grown in L broth supplemented with 100 μg of ampicillin per ml for 24 to 48 h at 37°C. The bacteria were then harvested by centrifugation at $10,000 \times g$ for 15 min, and the supernatant was retained for isolation of cell-free F1. The cell pellets were next resuspended in acetone, recentrifuged, and air dried for 15 h at room temperature. The dried organisms were extracted twice with toluene-saturated 2.5% NaCl overnight at 4°C. The reconstituted bacteria were then centrifuged, and the supernatants were pooled. Crude cell-associated capsular F1 from the salt extract supernatants was precipitated with ammonium sulfate added to 30% saturation. Protein that precipitated overnight at 4°C was collected by centrifugation at $13,000 \times g$ for 45 min, dialyzed against three changes of phosphate-buffered saline (PBS; pH 7.2), and passed across a 10-ml desalting column (Presto; Pierce, Rockford, Ill.) to remove any traces of ammonium sulfate. The preparations were then further enriched for F1 by adding ammonium sulfate to 25% saturation, which precipitated additional contaminating proteins. The partially pure F1 preparations, soluble in 25% ammonium sulfate, were then dialyzed extensively against PBS prior to column chromatography.

Cell-free F1 was extracted directly from the culture supernatants of both *E. coli* and *Y. pestis* broth cultures by a single 30% ammonium sulfate precipitation overnight at 4°C. The protein was collected by centrifugation, resuspended in 0.05× to 0.10× volume of PBS, and dialyzed against PBS. All fractions were assayed for total protein by the bicinchoninic acid-Lowry method (Pierce) and analyzed for the F1 subunit by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting (see below).

Chromatography. Sizing of the native F1 in partially pure preparations was determined by analytical gel filtration chromatography using a prepacked fast protein liquid chromatography Superose-6 column (Pharmacia Biotech, Inc., Piscataway, N.J.) with an exclusion limit of 4.0×10^7 Da. Fraction 1 antigen, obtained from 30% ammonium sulfate precipitates, was applied to the column at a flow rate of 0.2 ml/min in PBS (pH 7.2) or 50 mM diethanolamine (Sigma Chemical Co., St. Louis, Mo.) (pH 8.8).

Batch quantities of both *Y. pestis*- and *E. coli*-derived partially pure F1 were purified to homogeneity on a Superdex-200 Hiload preparative gel filtration column (Pharmacia) with a 300-ml bed volume and an exclusion limit of 1.3×10^6 Da. Five- to 10-ml volumes, containing 1 to 2 mg of the partially purified antigen per ml, were applied to the column at a flow rate of 2.0 ml/min in PBS (pH 7.2). Fractions (3 ml each) containing F1 were pooled, and the protein was precipitated with 50% ammonium sulfate for 15 h at 4°C. The precipitated protein was then centrifuged, concentrated 20-fold in PBS, and dialyzed extensively against PBS. Fractions from both columns were monitored for total protein by A_{280}, and F1 in each fraction was detected by immunoblotting with monoclonal antibody 3G8.

Analysis of pure F1 preparations and endotoxin removal. Both native and denaturing PAGE were performed on crude and purified preparations of F1, using Tris-glycine 4 to 20% polyacrylamide gradient gels (Novex, San Diego, Calif.). Samples run under denaturing conditions were resuspended in 1.0% SDS–2.5% β-mercaptoethanol (Sigma) and heated to 100°C for 10 min immediately before application on the gel. Denaturing gels were either stained with 0.5% Coomassie blue R-250 (Bio-Rad Laboratories, Hercules, Calif.) or silver stained after pretreatment with 0.7% periodic acid for 5 min (36). Samples run under native conditions were added directly to the sample wells of the gels without addition of SDS or heating.

F1 was immunologically detected by first electroblotting native or denaturing polyacrylamide gels onto nitrocellulose for 15 h at 100 mA in glycine-methanol buffer (31). Primary antibody was normally applied at dilutions of 1:200 to 1:1,000 for 3 to 12 h at room temperature, followed by blocking with 5% nonfat dry milk and 0.25% Tween 20 (Sigma) in PBS. After three washes with PBS–0.05% Tween 20 (PBST), a 1:500 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Boehringer Mannheim, Indianapolis, Ind.) was applied to the immunolabeled electroblots for 1.5 h. After two washes with PBST, the blots were developed for 1 to 5 min with 50 mM Tris (pH 8.0) containing 1 mg of naphthol AS-MX phosphate (Sigma) per ml and 2 mg of fast red TR salt (Sigma) per ml.

The isoelectric points of purified F1 antigens from both *Y. pestis* and *E. coli* were determined by IEF gels (pH 3 to 7) (Novex). IEF standards (Pharmacia) were run in parallel with all F1-containing samples. IEF gels were either stained with Coomassie blue R-250 after fixation in 10% trichloroacetic acid–3.5% sulfosalicylic acid (18) or transferred to nitrocellulose in the presence of 0.037% SDS. The IEF electroblots were then immunolabeled as described above.

Endotoxin levels of purified F1 preparations were estimated by the *Limulus* amoebocyte lysate assay (LALA). The procedure was followed essentially as described by the manufacturer, with *E. coli* O55:B5 LPS (Sigma) as a standard. Briefly, freeze-dried amoebocyte lysate from *Limulus polyphemus* (E-Toxate; Sigma) was reconstituted in pyrogen-free H_2O, and 100 μl was added into each of six sterile test tubes. Serial dilutions of F1 samples or endotoxin standard were next added to each tube. After 1 h of incubation at 37°C, approximate endotoxin levels were determined for each sample by inverting each set of dilution tubes and recording the lowest dilution of sample that failed to gel the lysate. This dilution was then compared with the endpoint from the dilution tube set of the *E. coli* standard. Contaminating endotoxin in F1 preparations was reduced by performing two to three passages of approximately 1.5 to 3.5 mg of chromatographed F1 across 1 ml of immobilized polymyxin B (Detoxi-Gel; Pierce) as recommended by the manufacturer. Alternatively, a column containing 30 ml of endotoxin-removing gel was used to effectively remove the bulk of contaminating endotoxin from milligram quantities of F1 by only a single chromatographic pass. Detoxified F1 preparations were then reassayed by LALA.

Preparation of F1 vaccines. Two days prior to animal immunizations, 500 to 1,000 μg of purified, detoxified *Y. pestis* and *E. coli* supernatant F1 (designated YpF1s and EcF1s, respectively) and cell-extracted F1 (YpF1c and EcF1c) were adsorbed to 1 to 2 ml of Alhydrogel in PBS overnight at 4°C with gentle rocking. Adsorption efficiencies for each preparation were calculated on the basis of the total amount of protein added to the adjuvant compared with the protein remaining in the supernatant after adsorption and centrifugation at $2,000 \times g$ for 5 min. After removal of the supernatant, the F1-Alhydrogel complex was adjusted to 50 μg of protein per ml with fresh sterile PBS. Mixtures of antigen and R-730 adjuvant were prepared by heating injection vials of 44 μl of R-730 emulsion to 45°C for 10 min before adding 2 ml of purified, detoxified F1 adjusted to 50 μg/ml.

Animal immunizations and virulent challenge. Groups of 10 female, 8- to 10-week-old outbred (Hsd:ND4) Swiss Webster mice (Harlan Sprague Dawley, Indianapolis, Ind.) were immunized with EcF1c, EcF1s, YpF1c, and YpF1s adsorbed to Alhydrogel or mixed with R-730 emulsion (two groups per antigen-adjuvant mixture). Two hundred microliters of each antigen-adjuvant mixture, containing 10 μg of F1, was administered at a single s.c. site on the backs of the animals. After 30 days, the animals were boosted with the identical dose and at the same inoculation site. Four negative control groups, consisting of 10 mice each, were immunized with the adjuvants only (two groups per adjuvant). Two positive control groups of 10 mice each were immunized similarly with two 200-μl doses of the licensed killed whole-cell plague vaccine. F1 antibody titers of all animal groups were measured 26 days after the second F1-immunizing dose

by a standard enzyme-linked immunosorbent assay (ELISA), with 200 ng of either *Y. pestis*-derived F1 or the recombinant antigen to coat each well of a 96-well flat-bottom microtiter plate.

The 11 immunized and control animal groups to receive the s.c. challenge were administered 100 50% lethal doses (LD_{50}) of wild-type *Y. pestis* CO92 (LD_{50} = 1.9 CFU) 28 days after the F1 booster dose. The second set of 11 animal groups was next exposed in a nose-only exposure chamber to a dynamic aerosol containing the virulent organisms (13, 26) diluted to give an inhaled dose of approximately 100 LD_{50} ($LD_{50} = 2.3 \times 10^4$ CFU). Samples of the aerosol were plated on blood agar to assay for the microorganisms, and the actual inhaled dose was determined by using Guyton's formula (17). All infected animals were monitored daily for disease symptoms and/or death until 28 days postchallenge. At day 28, surviving animals were bled for anti-F1 titer and euthanized, their spleens were cultured on blood agar base (Difco), and viable organisms were enumerated.

All animals were provided with feed and fresh water ad libitum during the entire course of this study. All animal experiments were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (10a).

Statistical analyses of protection levels between mouse groups immunized with F1 and those groups receiving adjuvant alone and between supernatant and cellular F1-immunized groups were made by the Fisher's exact test. The two-tail Fisher's exact test was used to evaluate differences between protection levels evoked by the killed whole-cell plaque vaccine and purified F1. Mean time to death values of the killed whole-cell plague vaccine groups and adjuvant-only groups were compared by Student's *t* test.

RESULTS

Expression of F1 in *Y. pestis* CO92 Pgm$^-$ Lcr$^-$ and the *E. coli* recombinant. Forty-eight- to 72-h broth cultures of the Pgm$^-$ Lcr$^-$ derivative of *Y. pestis* CO92 grown at 37°C yielded levels of F1 comparable to those produced by the wild type when examined by Western blotting (immunoblotting) (not shown). The capsule was efficiently secreted to the surface of the microorganism, as evidenced by India ink stain. The avirulent derivative of CO92 also displayed the typical temperature-regulated phenotype of F1, as evidenced by little or no capsule production when the organisms were grown at 30°C. On the basis of these observations, this avirulent variant of *Y. pestis* was selected as a suitable strain to extract capsular antigen in quantity from batch cultures.

F1 was initially cloned and expressed by Simpson and coworkers (33) from *E. coli* containing the recombinant plasmid pYPR1. When this strain was grown overnight in ampicillin-supplemented broth and stained with India ink, approximately 50% of the organisms did not express capsule, with many of the cells appearing elongated. Furthermore, a large number of cells lacked capsule when mid-log-phase cultures of this strain were examined. These observations suggested that *caf* gene expression was unstable in the original clone. We therefore created a new recombinant strain [*E. coli*(pBRF1); see Materials and Methods] which had more uniform capsule expression and cell length compared with the original clone. Additionally, as previously reported for *E. coli*(pYPR1) (33), the new F1 recombinant strain maintained the temperature-regulated F1 phenotype.

Extraction of F1 from cells and supernatants of broth cultures. A flowchart of the modified purification scheme for F1 antigen is shown in Fig. 1. This scheme differs from the original Baker method (4) by the substitution of liquid culture medium for agar and the application of two chromatographic steps (gel filtration and endotoxin removal) after two differential ammonium sulfate precipitations. Additionally, analysis of culture supernatants from both *Y. pestis* and *E. coli*(pBRF1) indicated that significant quantities (30 to 50%) of the antigen were released from the cells. This cell-free F1 could readily be precipitated and concentrated in partially pure state by a single application of ammonium sulfate to 30% saturation (Fig. 1).

Resuspending acetone-dried bacteria in toluene-saturated 2.5% NaCl and mild physical disruption effectively removed the bulk of the F1 capsule from the cells of both *Y. pestis* and *E. coli*(pBRF1). As shown by the SDS-polyacrylamide gel in Fig. 2, lane 1, a 30% ammonium sulfate precipitate of a representative salt extract from *Y. pestis* contained the 17-kDa F1 subunit as the major protein extracted from the cell surface. Almost all of the antigen was precipitated during this step, while significant amounts of contaminating proteins remained in solution (Fig. 2, lane 2). Similarly, high levels of F1 were easily removed from the surface of *E. coli*(pBRF1) cells, and adding ammonium sulfate to 30% saturation precipitated the bulk of the recombinant protein (not shown). After dialysis and desalting across a buffer exchange column (Fig. 1), *Y. pestis* and recombinant crude capsular F1 were further purified by adding 25% ammonium sulfate. In contrast to the 30% ammonium sulfate treatment, additional contaminating proteins were precipitated by using this step, while the majority of the F1 remained in solution (Fig. 2, lanes 3 and 4).

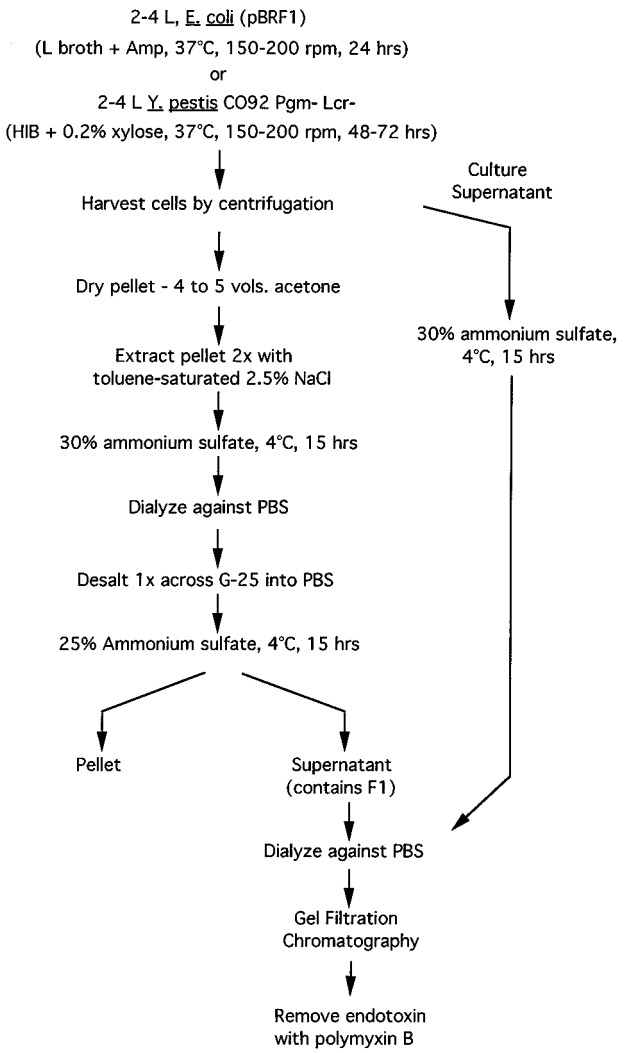

FIG. 1. Purification scheme for both *Y. pestis* F1 and the recombinant *E. coli*-derived protein. Amp, ampicillin.

Sizing of native F1, preparative gel filtration, and characterization of the final product. Native PAGE was performed on ammonium sulfate-precipitated, partially pure F1 in an effort to characterize the physical state of the protein to facilitate further purification. However, when the gel was stained for total protein, it was apparent that little, if any, undenatured F1 entered the gel. To detect low levels of F1 which may have

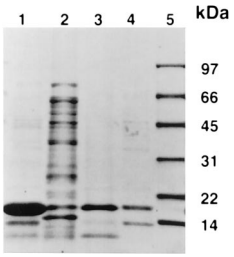

FIG. 2. Coomassie blue-stained 5 to 20% gradient SDS-polyacrylamide gel of *Y. pestis* cell-derived F1 enriched by differential precipitation with ammonium sulfate. Lanes: 1, protein precipitated from 30% ammonium sulfate treatment of salt extract followed by centrifugation and dialysis; 2, soluble protein remaining in supernatant after 30% ammonium sulfate treatment and centrifugation; 3, soluble protein after second salt precipitation (25%) and centrifugation; 4, precipitated protein after second salt treatment and centrifugation; 5, molecular mass markers. F1 was detected by immunoblotting (not shown) as the predominant 17-kDa band appearing in lanes 1 to 4. Positions of molecular mass markers are shown on the right.

penetrated the acrylamide matrix, a similar gel was electroblotted and immunolabeled with monoclonal antibody 3G8. A ladder-like pattern that corresponded to multimers of the 17-kDa F1 subunit ranging from 35 kDa to greater than 200 kDa was detected (not shown). Analytical gel filtration chromatography was therefore conducted on F1 to more precisely determine the size distribution of the antigen subunit aggregates. Shown in Fig. 3A, *Y. pestis* ammonium sulfate-precipitated cellular F1, applied across a Superose-6 column, was recovered predominantly as an aggregate of 2×10^6 Da. A much lower level of F1, in addition to other contaminating proteins, was detected in fractions which spanned a broad A_{280} peak at 1.5×10^5 Da. When supernatant-derived F1 was chromatographed under identical conditions, the same high-molecular-mass F1 aggregate was detected at 2×10^6 Da, as well as a minor broad A_{280} peak at 6.0×10^4 Da (Fig. 3B) which was composed of a mixture of low levels of F1 and contaminating proteins as detected by SDS-PAGE and immunoblot analyses (not shown). *E. coli*-derived F1 chromatographic profiles were identical to the *Y. pestis*-derived antigen. Additionally, the aggregative property of F1 from all sources remained unaltered when the pH of the column elution buffer was increased from 7.2 (PBS) to 8.8 (50 mM diethanolamine), as indicated by identical elution profiles which were obtained with the high-pH buffer. When fractions between A_{280} peaks from these chromatographic runs were examined by SDS-PAGE and immunoblotting, low levels of F1 were also detected (not shown).

The aggregative properties of F1 were next exploited to obtain larger quantities of the antigen through the use of preparative gel filtration chromatography by selecting a resin that would allow the isolation of F1 in the void volume of the column while retaining the contaminating proteins. Milligram quantities of cellular and supernatant F1 from *E. coli* or *Y. pestis* were applied to a Superdex-200 Hiload preparative gel filtration column. As analyzed by Western blotting, fractions containing the bulk of the F1 were recovered consistently at the exclusion limit of the column (1.3×10^6 Da), while contaminating protein species, as well as minor amounts of F1, were present in pooled fractions from the retained volume (not shown). A silver-stained SDS-polyacrylamide gel (Fig. 4) revealed that approximately 1 μg of protein (denatured) from the pooled and concentrated column void volumes from all four sources of F1 possessed a single species at 17 kDa with no other contaminating bands.

IEF was conducted on the purified products across a pH 3 to 7 gradient. A total protein stain of the IEF gel detected a diffuse band between an isoelectric point of 4.3 to 4.5 in all four F1 samples examined. Monoclonal antibody 3G8 was then used to probe an electroblot of an identical IEF gel. The bands from all four sources reacted strongly with the F1-specific antibody (not shown).

Final total yields of purified F1 from *Y. pestis* (YpF1c plus YpF1s) and *E. coli*(pBRF1) (EcF1c plus EcF1s) from a 2-liter broth culture were 35 and 375 mg/liter, respectively (Table 1). For purposes of comparison, yields of a representative F1 preparation from the original *E. coli* clone, *E. coli*(pYPR1) are also presented in Table 1. Equivalent dry weights of both recombinant strains from 2-liter cultures indicated that the stabilized F1 construct, *E. coli*(pBRF1), produced approximately threefold-higher levels of the antigen from cellular extracts as well as from the culture supernatants compared with *E. coli*(pYPR1). More strikingly, the new recombinant strain produced 10-fold-higher total F1 yields compared with *Y. pestis*. Supernatant-derived F1 from *Y. pestis* and both *E. coli*

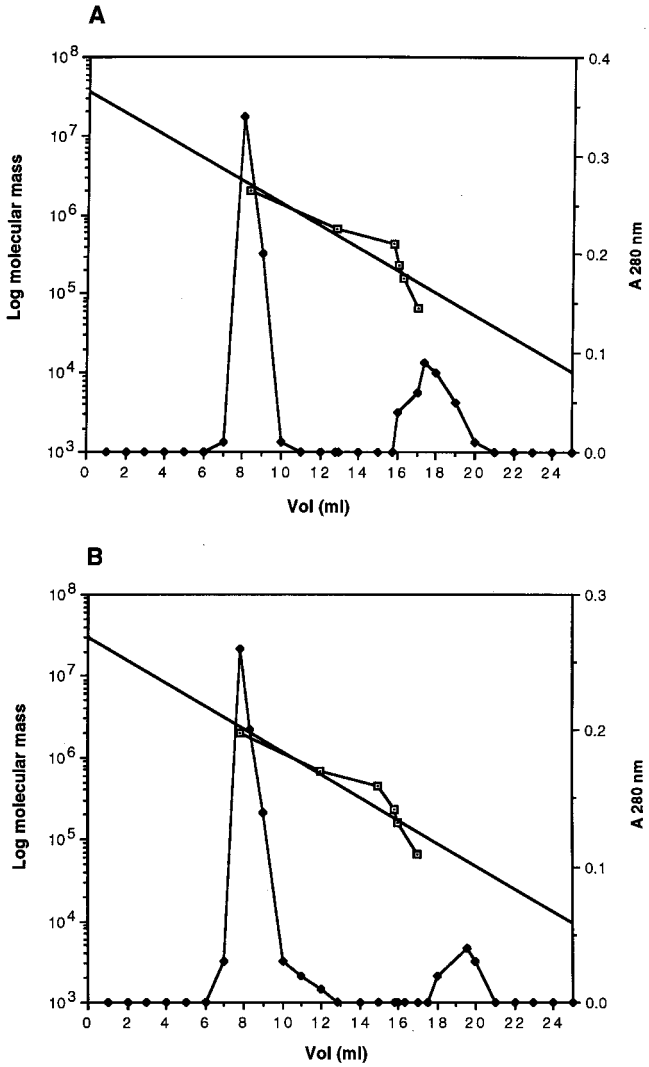

FIG. 3. Analytical gel filtration chromatography of *Y. pestis*-derived crude cellular F1 (A) and cell-free F1 (B). Regression lines drawn across both *y* axes were derived from the molecular mass calibration curve. ◆, A_{280}; ⊡, molecular mass markers. Identical chromatographic profiles were obtained with the recombinant protein (not shown).

FIG. 4. Silver-stained gradient SDS-polyacrylamide gel (5 to 20%) of F1 preparations after preparative gel filtration across Superdex-200. Lanes: 1, cell-derived *E. coli* recombinant F1; 2, cell-free recombinant F1; 3, cell-derived *Y. pestis* F1; 4, cell-free *Y. pestis* F1. All lanes were loaded with approximately 1 μg of purified protein.

recombinant strains also represented >50 and 30% of the total F1, respectively (Table 1).

Despite the apparent purity of the chromatographed F1 preparations, significant endotoxin levels were detected in F1 from all sources by the LALA. Purified F1 preparations were therefore passed across immobilized polymyxin B to adsorb the contaminating endotoxin. As shown in Table 1, >90% of the endotoxin in *Y. pestis* F1 was removed after passage across the column. Much higher levels of endotoxin detected in the *E. coli* (pBRF1) recombinant antigen preparations were similarly reduced.

Lethal challenge of mice immunized with purified F1. After detoxification, the highly purified F1 preparations were adsorbed to Alhydrogel or mixed with R-730 emulsion. By analysis of postadsorption adjuvant supernatants, adsorption of F1 to Alhydrogel, in all cases, was >85%. All four F1 preparations were then used in combination with the two adjuvants to immunize 16 groups of outbred mice before challenge with a lethal dose of *Y. pestis* CO92.

As indicated in Table 2, animals immunized with *Y. pestis* or *E. coli*(pBRF1)-derived F1 were protected from a lethal s.c. challenge, with 70 to 100% survival in the different groups (Table 2). Survival within each F1-immunized animal group was independent of the source of the antigen, whether cell derived or cell free, from either *Y. pestis* or *E. coli*, and the adjuvant used ($P = 0.211$ to 1.00). There was decreased survival in the licensed killed whole-cell plague vaccine group (50% [5 of 10]) compared with the F1-immunized groups (91% [73 of 80]; $P = 0.0032$), although mice succumbing to the infection possessed a mean time to death of >20 days, compared with 5 days for animals immunized with the adjuvants alone (Table 2, $P = 0.0016$). Only 2 of the 78 spleens removed from animals surviving infection yielded cultures positive for *Y. pestis* (Table 2).

When recombinant F1-immunized mice were challenged by the aerosol route, 84% (32 of 38) of the animals survived, while 65% (26 of 40) of all *Y. pestis*-derived F1-immunized animals survived aerosol infection (Table 3). Although it appeared that the recombinant F1 was slightly more effective at eliciting a protective response, the difference in protection between the two sets of immunized animals was not statistically significant ($P = 0.07$). However, only 11% (1 of 9) of the killed whole-cell plague vaccine-immunized mice exposed to aerosolized organisms survived, which was significantly lower than the survival of groups immunized with purified F1 (74% [58 of 78]; $P = 0.00037$). While the killed whole-cell vaccine had little effect on survival, there was a significant prolongation of the time to death of the vaccine-immunized animals compared with animals treated with adjuvants alone (Table 3, $P = 0.011$). As with the s.c.-challenged immunized animals, there were no differences in protection from aerosol challenge with F1 combined with either of the two adjuvants or with supernatant- versus cell-derived antigen ($P = 0.559$). Only 2 of 59 animals had positive spleen cultures.

Figure 5 shows the reciprocal geometric mean F1 antibody titers as determined by ELISA for pooled serum samples from each mouse group 56 days after the first immunization (2 days before s.c. challenge) and 28 days postchallenge (survivors). As shown in Fig. 5, all F1-immunized groups possessed very high antibody titers to F1. F1 titers of the killed whole-cell vaccine-

TABLE 1. Total yields of pure F1 from broth cultures of *Y. pestis* and *E. coli* recombinant strains

Source of F1	F1 yield (mg/liter)	Endotoxin level[a]	
		Preadsorbed	Postadsorbed
Y. pestis			
Whole cells (YpF1c)	16	2,500	147
Supernatant (YpF1s)	19	282	4
E. coli(pBRF1)			
Whole cells (EcF1c)	240	12,800	200
Supernatant (EcF1s)	135	24,600	13
E. coli(pYPR1)			
Whole cells	76	ND	ND
Supernatant	40	ND	ND

[a] Expressed as endotoxin units per milligram of F1 pre- and postadsorbed with polymyxin B as determined by LALA. One endotoxin unit is 0.1 ng of *E. coli* O55:B5 LPS standard. ND, not determined.

TABLE 2. Survival of mice after s.c. challenge with 100 LD_{50} of wild-type *Y. pestis* CO92[a]

Treatment group	No. of survivors[a]	Mean time to death (days) ± SD	No. of culture-positive spleens/total
R-730 only	0	5.3 ± 1.0	
Alhydrogel only	0	4.9 ± 0.7	
Plague vaccine	5	20.6 ± 4.6	0/5
EcF1c + R-730	10		1/10
EcF1s + R-730	10		1/10
EcF1c + Alhydrogel	7	15.3 ± 3.5	0/7
EcF1s + Alhydrogel	10		0/10
YpF1c + R-730	10		0/10
YpF1s + R-730	7	10.3 ± 4.2	0/7
YpF1c + Alhydrogel	10		0/10
YpF1s + Alhydrogel	9	11.0 ± 0.0	0/9

[a] In each case, 10 mice were examined.

TABLE 3. Survival of mice after aerosol challenge with 100 LD_{50} of wild-type *Y. pestis* CO92

Treatment group	No. of survivors/total	Mean time to death (days) ± SD	No. of culture-positive spleens/total
R-730 only	0/10	3.3 ± 0.5	
Alhydrogel only	0/10	3.3 ± 0.7	
Plague vaccine	1/9	11.8 ± 7.0	0/1
EcF1c + R-730	9/10	6.0 ± 0.0	0/9
EcF1s + R-730	6/8	17.0 ± 2.8	0/6
EcF1c + Alhydrogel	8/10	14.0 ± 12.2	0/8
EcF1s + Alhydrogel	9/10	10.0 ± 0.0	0/9
YpF1c + R-730	6/10	8.5 ± 5.1	0/6
YpF1s + R-730	6/10	9.8 ± 5.2	0/6
YpF1c + Alhydrogel	7/10	13.3 ± 11.9	0/7
YpF1s + Alhydrogel	7/10	12.0 ± 11.4	2/7

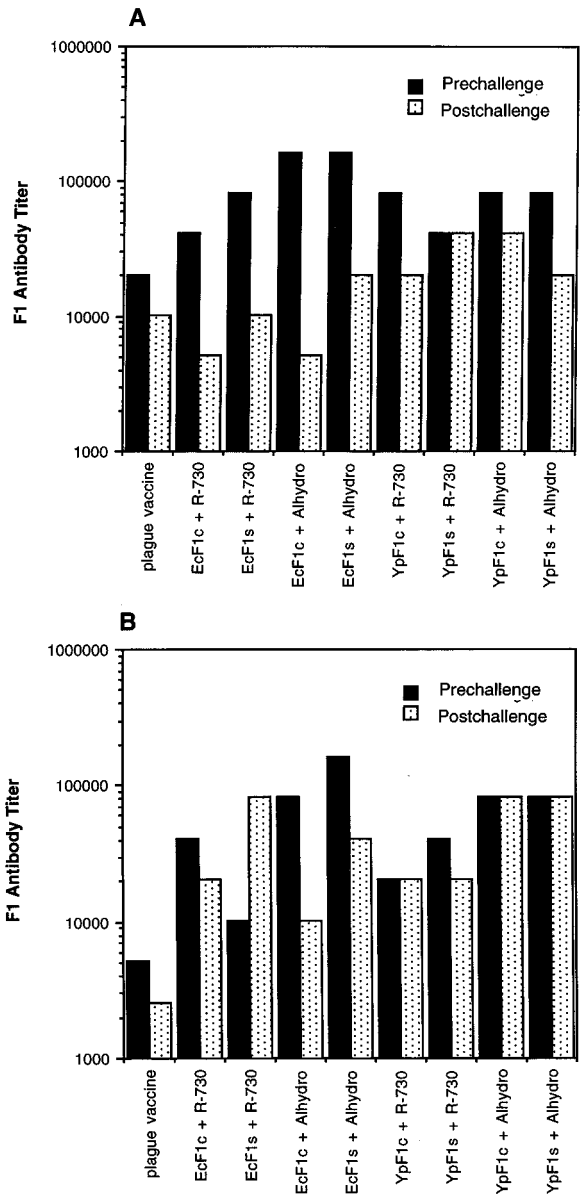

FIG. 5. Reciprocal geometric mean F1 antibody titers of pooled serum samples from mouse groups immunized with plague vaccine and purified F1, challenged s.c. (A) or by aerosol (B). Anti-F1 titers for Alhydrogel (Alhydro) and R-730 adjuvants only for both sets of challenged groups were negligible and are not shown.

immunized groups, while measurable, were lower than in the animals receiving purified F1. Pooled sera from animals surviving lethal s.c. challenge had mean F1 antibody titers which were lower than or unchanged from their prechallenge antibody levels (Fig. 5A). Similarly, mean antibody titers from survivors of the aerosol challenge were unchanged or reduced in eight of the nine groups (Fig. 5B).

DISCUSSION

Although the Baker extraction method with agar medium (4) has long been used for preparation of F1 from *Y. pestis*, some studies found that *Y. pestis* grown in liquid culture medium is capable of producing 50 to 1,000 mg of F1 per liter (12, 37). While yields of F1 from *Y. pestis* grown in liquid culture in our laboratory were lower, they were comparable to previously reported results on the levels of antigen extracted from the agar-grown organisms (4). Thus, growth of *Y. pestis* in liquid culture represents a more practical approach to scale-up production of large quantities of the antigen and was incorporated into our extraction and purification scheme (Fig. 1). Further, the use of the avirulent Pgm^- Lcr^- derivative of *Y. pestis* CO92 facilitated safe handling of liter quantities of broth cultures. Interestingly, however, we found that *E. coli* recombinant strains expressed F1 in liquid culture constitutively during log phase growth, whereas a delay of approximately 12 to 24 h was noted with expression of the antigen from *Y. pestis* (2). This observation, in addition to the much higher levels of F1 produced from our stabilized *caf* clone, *E. coli*(pBRF1), favors this recombinant strain for scale-up production of the antigen.

A previous report of the release of F1 from the cell surface in liquid culture medium (12) was confirmed by our findings. This phenomenon was advantageous for our extraction and purification scheme, as cell-free F1 could be isolated and purified more easily than the cell-bound antigen, hence simplifying adaptation of the purification procedure for scale-up. Furthermore, supernatant-derived F1 was not exposed to organic solvents (as was the cell-bound form) during extraction and purification, which may have altered immunogenicity of the protein.

Despite the much greater purity of our F1 preparations than of early crude capsular extracts, analysis by LALA revealed endotoxin contamination in all of the F1 preparations. We surmised that early studies attempting to clarify the importance of F1 as a protective antigen were likely compromised by the presence of this immunomodulatory outer membrane component present in crude capsule preparations. In fact, our findings indicated that endotoxin levels in crude preparations of F1 made in other laboratories by the Baker method (4) ranged between 2,500 and 5,200 endotoxin units/mg of protein (19). Further, the possibility of endotoxin contamination in the F1 preparation used by Simpson et al. (33) was not addressed. Therefore, to preclude endotoxin involvement in protection in our study, all four F1 preparations were detoxified. Although traces of endotoxin remained in the F1 preparations after this procedure, endotoxin levels received by mice upon immunization were extremely low (<0.2 ng of endotoxin per F1 dose of 10 μg).

Given the vastly improved quality of the new F1 preparations, their efficacy as protective immunogens could be more clearly evaluated in vivo. F1 antigen from all four sources evoked a high degree of protection in animals challenged s.c. with 100 LD_{50} of wild-type *Y. pestis*. Protection also appeared to be independent of the adjuvant used. Previous studies used various adjuvants with F1 (30, 41). Our results, however, suggest that F1 combined with Alhydrogel, the only adjuvant approved for human use, elicits good protective immunity at the challenge dose administered.

Although not achieving the level of protection seen against s.c. challenge, F1 from all four sources also protected the majority of immunized mice against an aerosol challenge of 100 LD_{50}. These results confirm early reports of partial protection against aerosolized *Y. pestis* in rodent models (23, 39). In contrast, data in some studies derived from aerosol infection of F1-immunized nonhuman primates suggested poor protection (34), while others demonstrated good protection against experimental aerosol infection in monkeys by hyperimmunization (11) or use of more potent adjuvants (30). Despite the variability seen in protection elicited by F1 immunization against pneumonic plague in past studies, the degree of pro-

tection (against parenteral challenge as well) appears to be correlated with F1 antibody levels (39), although the response to potential contaminants such as LPS was not measured. In this study, although all F1-immunized mice possessed very high anti-F1 antibody in their pooled sera, mortality did occur in some animals, but lower titers measured in some individual animals did not appear to correlate with these deaths. One explanation of this observation may be that even though high levels of F1 antibody are present, cell-free F1 may bind significant quantities of this circulating immunoglobulin, titrating out its activity. Alternatively, cell-associated F1 which has bound antibody may be sloughed from the bacteria as has been described for some antibody-bound streptococcal surface antigens (24). These immune-avoidance hypotheses are consistent with our observation that the bulk of the F1 is released from the microorganism in vitro after an extended growth period. Furthermore, immunoelectron microscopic analysis of infected mouse tissue has shown F1 capsular material in areas devoid of bacteria (data not shown). Higher overall mortality levels seen in aerosol-challenged F1-immunized mice were observed despite high levels of serum antibody. In this regard, interaction of circulating antibody with the microorganism may have been compromised by the capillary endothelial-alveolar epithelial interface, since serum immunoglobulin must transudate this blood-air barrier (6).

We observed a lower level of protection (5 of 10 survivors) against s.c. challenge of animals immunized with the commercial killed whole-cell plague vaccine compared with that in the F1-immunized groups. Additionally, plague vaccine-immunized mice challenged by aerosolized organisms were not significantly protected against death ($P = 0.474$). Further, the lower level of protection in plague vaccine-immunized groups was associated with a lower pooled serum titer to F1 (Fig. 5). Despite these findings, estimates of the levels of antigen contained in 200 μl of the commercial vaccine preparation suggest that mice in these groups actually received two- to threefold-higher levels of F1 (20 to 30 μg) than received by mice in the F1-immunized groups (2). One explanation for the reduced efficacy of the killed whole-cell vaccine is that irreversible alteration of critical protective epitopes of F1 may occur during the preparation of the vaccine, since the vaccine strain, *Y. pestis* 195P, is killed by exposure to formalin (27). Long-term storage of the vaccine (>4 months) at 4°C may also contribute to reduced protective efficacy of F1, since one laboratory reported a breakdown and subsequent decrease of immunochemical activity of the antigen under these storage conditions (37). In fact, analysis of plague vaccine-derived F1 by analytical gel filtration chromatography in our laboratory indicated that the bulk of the antigen existed in lower-molecular-mass aggregates, with the larger aggregate representing only a minor species (2). This finding is interesting in light of the fact that protective immunogenicity of other bacterial antigens, such as Vi antigen of *Salmonella* species, is a function of molecular mass (25). Alternatively, differences in the protective immunogenicity of the plague vaccine may be simply due to the absence of any added adjuvant.

Despite sterile immunity in the vast majority of surviving animals, *Y. pestis* was cultured from splenic tissues of four animals. Interestingly, one isolate was negative for F1 expression as a result of genetic alteration of regulatory sequences within the F1 operon (1). The mechanisms whereby the organisms maintain themselves within these few isolated mice are unknown, although cryptic plague infection in other animal models has been reported (9). It is quite possible, therefore, that additional genetic alterations which allowed these isolates to persist in the spleen may have occurred during the course of infection. We are presently exploring this hypothesis in our laboratory.

Finally, the general observation that some F1-immunized animals succumbed to infection by either challenge route suggests the requirement for immunity to additional virulence determinants for complete protection against plague, particularly against the pneumonic form of the disease. We are therefore currently examining the roles of other purified *Y. pestis* virulence proteins, individually and combined with F1, in protection against plague for use in a new multicomponent plague vaccine.

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REFERENCES

1. **Anderson, G. W., Jr., P. L. Worsham, G. P. Andrews, C. R. Bolt, S. L. Welkos, A. M. Friedlander, and J. P. Burans.** 1995. Passive immunization with monoclonal antibodies against the F1 antigen of *Yersinia pestis* protects mice from fatal bubonic and pneumonic plague, abstr. 211. *In* Abstracts from the 44th Annual Meeting of the American Society of Tropical Medicine and Hygiene.
2. **Andrews, G. P.** Unpublished observations.
3. **Atlas, R. M., A. E. Brown, K. W. Dobra, and L. Miller (ed.).** 1984. Experimental microbiology: fundamentals and applications, p. 54. Macmillan Publishing Company, New York.
4. **Baker, E. E., H. Sommer, L. E. Foster, E. Meyer, and K. F. Meyer.** 1952. Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. J. Immunol. **68:**131–145.
5. **Bennett, L. G., and T. G. Tornabene.** 1974. Characterization of the antigenic subunits of the envelope protein of *Yersinia pestis*. J. Bacteriol. **117:**48–55.
6. **Bienenstock, J. (ed.).** 1984. Immunology of the upper respiratory tract, p. 159–163. McGraw-Hill Book Company, New York.
7. **Bolivar, F., R. L. Rodriguez, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
8. **Cavanaugh, D. C., B. L. Elisberg, C. H. Llewellyn, J. D. Marshall, Jr., J. H. Rust, Jr., J. E. Williams, and K. F. Meyer.** 1974. Plague immunization. V. Indirect evidence for the efficacy of plague vaccine. J. Infect. Dis. **129:**S37–S40.
9. **Cavanaugh, D. C., and J. E. Williams.** 1977. Plague: some ecological interrelationships, p. 245–256. *In* R. Traub and H. Starcke (ed.), Fleas. A. A. Balkema, Rotterdam, The Netherlands.
10. **Chen, T. H., and S. S. Elberg.** 1977. Scanning electron microscopic study of virulent *Yersinia pestis* and *Yersinia pseudotuberculosis* type 1. Infect. Immun. **15:**972–977.

10a. **Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.** 1985. Guidelines for the care and use of laboratory animals, revised. National Institutes of Health publication no. 86-23. National Institutes of Health, Bethesda, Md.

11. **Ehrenkanz, N. J., and K. F. Meyer.** 1955. Studies on the immunization against plague. VIII. Study of three immunizing preparations in protecting primates against plague. J. Infect. Dis. **96:**138–144.
12. **Englesberg, E., and J. B. Levy.** 1954. Studies on the immunization against plague. VI. Growth of *Pasteurella pestis* and the production of the envelope and other soluble antigens in a casein hydrolysate mineral glucose medium. J. Bacteriol. **67:**438–449.
13. **Estep, J. E., M. L. M. Pitt, S. L. Welkos, K. Davis, and A. Friedlander.** 1994. Pneumonic plague: development of rodent animal models using nose-only aerosol exposure, abstr. B-212. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
14. **Galyov, E. E., A. V. Karlishev, T. V. Chernovskaya, D. A. Dolgikh, O. Y. Smirnov, K. I. Volkovoy, V. M. Abramov, and V. P. Zav'yalov.** 1991. Expression of the envelope antigen F1 of *Yersinia pestis* is mediated by the product of caf1M gene having homology with the chaperone protein PapD of *Escherichia coli*. FEBS Lett. **286:**79–82.
15. **Galyov, E. E., O. Y. Smirnov, A. V. Karlishev, K. I. Volkovoy, A. I. Denesyuk, I. V. Nazimov, K. S. Rubtsov, V. M. Abramov, S. M. Dalvadyanz, and V. P. Zav'yalov.** 1990. Nucleotide sequence of the *Yersinia pestis* gene encoding F1 antigen and the primary structure of the protein. FEBS Lett. **277:**230–232.
16. **Gremyakina, T. A., V. N. Stephanshina, O. V. Korobova, and G. A. Anisimov.** 1994. Protective activity of *Salmonella minnesota* R595/GSA recombinant

strain synthesizing *Yersinia pestis* capsular antigen in experimental plague in mice. Mol. Gen. Mikrobiol. Virusol. **7:**23–26. (In Russian.)
17. **Guyton, A. C.** 1947. Measurement of the respiratory volumes of laboratory animals. Am. J. Physiol. **150:**70–77.
18. **Hanes, B. D., and D. Rickwood (ed.).** 1990. Gel electrophoresis of proteins: a practical approach, 2nd ed. IRL Press, Oxford.
19. **Heath, D. G.** Unpublished observations.
20. **Higuchi, K., and J. L. Smith.** 1961. Studies on the nutrition and physiology of *Pasteurella pestis*. VI. A differential plating medium for estimation of the mutation rate to avirulence. J. Bacteriol. **81:**605–608.
21. **Karlyshev, A. V., E. E. Galyov, V. M. Abramov, and V. P. Zav'yalov.** 1992. *caf1R* gene and its role in the regulation of capsule formation of *Yersinia pestis*. FEBS Lett. **305:**37–40.
22. **Karlyshev, A. V., E. E. Galyov, O. Y. Smirnov, A. P. Guzayev, V. M. Abramov, and V. P. Zav'yalov.** 1992. A new gene of the f1 operon of *Y. pestis* involved in the capsule biogenesis. FEBS Lett. **297:**77–80.
23. **Lawton, W. D., G. M. Fukui, and M. J. Surgalla.** 1960. Studies on the antigens of *Pasteurella pestis* and *Pasteurella pseudotuberculosis*. J. Immunol. **84:**475–479.
24. **Lee, S. F.** 1995. Active release of bound antibody by *Streptococcus mutans*. Infect. Immun. **63:**1940–1946.
25. **Martin, D. G., F. G. Jarvis, and K. C. Milner.** 1967. Physiochemical and biological properties of sonically treated Vi antigen. J. Bacteriol. **94:**1411–1416.
26. **May, K. R.** 1973. The Collison nebulizer: description, performance and applications. J. Aerosol Sci. **4:**235–243.
27. **Meyer, K. F.** 1970. Effectiveness of live or killed plague vaccines in man. Bull. W.H.O. **42:**653–666.
28. **Meyer, K. F., J. A. Hightower, and F. R. McCrumb.** 1974. Plague immunization. VI. Vaccination with the fraction 1 antigen of *Yersinia pestis*. J. Infect. Dis. **129:**S41–S45.
29. **Oyston, P. C. F., E. D. Williamson, S. E. C. Leary, S. M. Eley, K. F. Griffin, and R. W. Titball.** 1995. Immunization with live recombinant *Salmonella typhimurium aroA* producing F1 antigen protects against plague. Infect. Immun. **63:**563–568.
30. **Pautov, V. N., Y. V. Chicherin, V. I. Yevstigneyev, A. A. Byvalov, and O. A. Kedrov.** 1979. Experimental study of protective properties of fraction I isolated from plague bacterium. Zh. Mikrobiol. Epidemiol. Immunobiol. **12:**37–42. (In Russian.)
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis (ed.).** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Serdobintsev, L. N., T. M. Taranenko, A. V. Naumov, I. P. Andreeva, A. A. Shcherbakov, and K. M. Mosin.** 1982. Preparation of capsular antigen of *Yersinia pestis* by ion-exchange chromatography on DEAE cellulose, p. 38–43. *In* A. V. Gor'kova (ed.), Immunoliya i profilaktika osobo opasnykh infektsii. Microb, Saratov, Russia. (In Russian.)
33. **Simpson, W. J., R. E. Thomas, and T. G. Schwan.** 1990. Recombinant capsular antigen (fraction 1) from *Yersinia pestis* induces a protective antibody response in BALB/C mice. Am. J. Trop. Med. Hyg. **43:**389–396.
34. **Speck, R. S., and H. Wolochow.** 1957. Studies on the experimental epidemiology of respiratory infections. VIII. Experimental pneumonic plague in *Macacus rhesus*. J. Infect. Dis. **100:**58–69.
35. **Surgalla, M. J., and E. D. Beesley.** 1969. Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. Appl. Microbiol. **18:**834–837.
36. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
37. **Veynblat, V. I., M. M. Titenko, M. S. Verenkov, P. I. Men'shov, A. V. Kormilitsyn, O. T. Mozharov, A. A. Shcherbakov, A. S. Vasenin, and S. Y. Zadumina.** 1985. Characterization of samples of capsular antigen separated from broth cultures of the EV strain of *Yersinia pestis*. Zh. Mikrobiol. Epidemiol. Immunol. **4:**19–23. (In Russian.)
38. **Vorontsov, E. D., A. G. Dubichev, L. N. Serdobintsev, and A. V. Naumov.** 1990. Association-dissociation processes and supermolecular organization of the capsule antigen (protein F1) of *Yersinia pestis*. Biomed. Sci. **1:**391–396.
39. **Williams, J. E., and D. C. Cavanaugh.** 1979. Measuring the efficacy of vaccination in affording protection against plague. Bull. W.H.O. **57:**309–313.
40. **Williams, R. C., H. Gewurz, and P. G. Quie.** 1972. Effects of fraction I from *Yersinia pestis* on phagocytosis in vitro. J. Infect. Dis. **126:**235–241.
41. **Zakrevskiy, V. I., and N. G. Plekhanova.** 1990. Study of protective properties of antigen-containing liposomes of varying lipid composition in plague. Vestn. Akad. Med. Nauk SSSR **8:**41–44. (In Russian.)

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