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# Effective Protective Immunity to *Yersinia pestis* Infection Conferred by DNA Vaccine Coding for Derivatives of the F1 Capsular Antigen

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**Three plasmids expressing derivatives of the *Yersinia pestis* capsular F1 antigen were evaluated for their potential as DNA vaccines. These included plasmids expressing the full-length F1, F1 devoid of its putative signal peptide (deF1), and F1 fused to the signal-bearing E3 polypeptide of Semliki Forest virus (E3/F1). Expression of these derivatives in transfected HEK293 cells revealed that deF1 is expressed in the cytosol, E3/F1 is targeted to the secretory cisternae, and the nonmodified F1 is rapidly eliminated from the cell. Intramuscular vaccination of mice with these plasmids revealed that the vector expressing deF1 was the most effective in eliciting anti-F1 antibodies. This response was not limited to specific mouse strains or to the mode of DNA administration, though gene gun-mediated vaccination was by far more effective than intramuscular needle injection. Vaccination of mice with deF1 DNA conferred protection against subcutaneous infection with the virulent *Y. pestis* Kimberley53 strain, even at challenge amounts as high as 4,000 50% lethal doses. Antibodies appear to play a major role in mediating this protection, as demonstrated by passive transfer of anti-deF1 DNA antiserum. Taken together, these observations indicate that a tailored genetic vaccine based on a bacterial protein can be used to confer protection against plague in mice without resorting to regimens involving the use of purified proteins.**

*Yersinia pestis*, the causative agent of plague, still represents a serious public health threat in various regions of the world and at the same time is gaining attention as a potential agent in bioterrorism. Even though live and killed whole-cell vaccines are available for human use, serious drawbacks limit their use for prevention of natural or human-inflicted outbreaks (22, 23, 39). Two *Y. pestis* proteins, F1 and V, are known to be effective immunogens and have been proposed as candidates for a combined subunit vaccine against plague (2, 38).

The fraction 1 capsular protein (F1), which is encoded by the 100-kb pFra plasmid, forms a large gel-like capsule containing multimeric F1 aggregates (7). The F1 gene was found to code for a 17.5-kDa polypeptide carrying a putative secretion signal (16). F1 is considered an important but not essential virulence factor unique to *Y. pestis* (12, 37). Deletion of the F1 gene does not abolish virulence but leads to a delay in onset of the disease in animal models.

F1 appears to have a role in blocking uptake by macrophages (13), yet its exact function in this respect is not clear. Interestingly, the structural gene of F1 has been shown to be homologous to interleukin 1β (IL-1β) and has been suggested to interact with IL-1 receptors (1). Such interactions may indicate that F1 participates in early stages of plague development and regulates the contact of the bacteria with the host. This could explain the high efficiency of anti-F1 antibodies in blocking infection.

F1 is an extremely immunogenic protein in both animals and humans. Immunization with multiple doses of F1 has been shown to protect mice against subcutaneous challenge with wild-type *Y. pestis* (3, 38), and a combined formulation containing F1 and V antigen confers protection against airborne infection (39). The protein has been associated with elicitation of protective immune response in humans as well (24). Hyperimmune sera from F1-immunized volunteers possessed F1 antibodies that can passively protect mice from virulent plague challenge.

The observation that genetic immunization is able to elicit protective immunity (33) has fostered the development of a new generation of vaccines. DNA vaccines provide prolonged antigen expression, leading to amplification of the immune response, and appear to offer certain advantages, such as ease of construction, low cost of mass production, high levels of temperature stability, and the ability to elicit both humoral and cell-mediated immune responses (for recent reviews, see references 20 and 26). The endogenous expression of antigen from DNA introduced into host cells leads to peptide presentation with the major histocompatibility complex class I (MHC-I), which is ideal for induction of cytotoxic T-cell response. Therefore, DNA vaccines have been primarily considered for use against intracellular pathogens such as viruses (18, 27). Nevertheless, the observed ability of DNA vaccines to elicit both cell-mediated and humoral immune responses paved the way for their assessment as expressers of soluble, secreted bacterial antigens, conferring immunity presumably by eliciting the classical MHC-II-mediated humoral response. The efficacy of such DNA vaccines was found to vary from case to case and depended on the nature of the individual antigen, on the vaccination mode (15), and on the subcellular location in which the antigen was expressed (8, 32, 36).

In a previous attempt to develop genetic vaccination against *Y. pestis* by using F1 DNA, it was found that outbred mice were

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nonresponsive and inbred mice gave a weak anamnestic response (9). The advances in genetic vaccination and the accumulating information on factors that modulate the extent of response to DNA vaccines led us to reexamine genetic vaccination based on F1 antigen.

In this report, we compare three F1 DNA derivatives carrying different signals for cellular localization and demonstrate that one such genetic derivative, which presumably targets expression to the cytosol, induces an effective antibody response and confers protection against high doses of infective *Y. pestis.*

## MATERIALS AND METHODS

**Cloning of F1 derivatives and construction of expression plasmids.** All constructs are based on the eukaryotic expression vector pCI (Promega), which carries the efficient eukaryotic cytomegalovirus promoter, a recombinant chimeric intron, the prokaryotic T7 promoter, the late simian virus 40 polyadenylation signal, and an ampicillin resistance marker.

The *Y. pestis caf1* gene (GenBank accession no. X61996) was cloned by PCR from the DNA of the virulent strain Kimberley53. For cloning of the full-length F1 gene, primers nF1 (ACTGCAGTCCACCCACCATGAAAATCAGTTCCG TTATCGCC) and cF1 (TCATCGGCGGCCGCCTATTATTGGTTAGATAC GGTTACGG) were used. To generate an F1 derivative (deF1) lacking the putative bacterial signal peptide (16), primers nF2 (ACTGCAGTCCACCCACC ATGGCAGATTTAACTGCAAGCACC) and cF1 were used. The two resulting PCR products were digested by *Sal*I and *Not*I and ligated to pCI plasmid linearized by the same restriction enzymes, yielding the pCI-F1 and pCI-deF1 expression vectors.

The Semliki Forest virus (SFV) E3-*Y. pestis* F1 fusion protein product (E3/F1) was generated as follows. The SFV E3 gene (198 bp, coding for 66 amino acids) was derived from a plasmid carrying SFV DNA (17, 19) as a PCR product using primers nE (CTCACAAAGCTAGCCACCATGTCCGCCC CGCTGATTAC TG) and cE (CTCACGAATTCGGAGGCCTCCGGTGTCTTGTT CCGTTTC G). This fragment was then inserted into pCI between the *Nhe*I and *Stu*I sites. The F1 sequence was inserted between the *Stu*I and *Sal*I sites to obtain pCI-E3/F1. All recombinant plasmids were verified by restriction enzyme analysis and sequencing.

**In vitro translation of F1 derivatives.** The coupled reticulocyte lysate in vitro transcription and translation system (TNT; Promega) was used as recommended by the manufacturer to generate $^{35}$S-labeled F1 derivatives. Translocation of the newly formed, labeled polypeptides into membranal fractions was examined by adding canine microsomes (Promega). Where indicated, translation products were treated with proteinase K (0.2 mg/ml; Sigma) for 15 min at 4°C in the presence or absence of 1% Triton X-100. The in vitro translated proteins were resolved by sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis (PAGE). Gels were fixed in 10% acetic acid–30% methanol for 12 h, soaked in Enhance solution (Du Pont) for 30 min, washed in water for 30 min, and dried on Whatman 3MM filter paper at 80°C. Gels were then exposed to Kodak X-Omat AR film at −70°C for the indicated times. $^{14}$C-labeled protein molecular weight (MW) markers (Rainbow High MW; Amersham) were included as protein size markers.

**Transfection and visualization of expressed antigens.** For visualization by immunofluorescence staining, recombinant plasmids were used to transiently transfect HEK293 cells (34) in individual wells of a Permonax chamber slide system (Nunc International) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. At 24 h posttransfection, cells were fixed with acetone for 20 min at −20°C. The fixed cells were incubated with polyclonal rabbit anti-F1 antibodies (1:1,000 dilution) for 60 min, washed three times, and treated for 60 min with goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (Sigma). Immunolabeled cells were viewed with a Zeiss fluorescence microscope.

For visualization by Western immunoblotting, transiently transfected HEK293 cells were detached, washed with phosphate-buffered saline (PBS), and resuspended in 0.025 M Tris, pH 8.0. Cells were disrupted by three rounds of freeze-thaw. Following centrifugation at 14,000 rpm in an Eppendorf microcentrifuge, cleared lysates were collected. Cell extracts were stored at −20°C until further use. Ten-microliter aliquots were resolved by SDS–12.5% PAGE and electroblotted onto nitrocellulose membranes. Blots were probed for 60 min with rabbit polyclonal anti-F1 antiserum (1/1,000 dilution in skimmed milk) followed by a 60-min incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1/2,000; Sigma). Bound peroxidase was visualized by chemiluminescence (Pierce SuperSignal kit).

**Metabolic labeling and immunoprecipitation.** Metabolic labeling of HEK293 cells was performed essentially as described previously (21). Twenty-four hours posttransfection, cells were washed with fresh medium without methionine and incubated in the same medium for an additional 1 h. Medium containing [$^{35}$S]methionine (0.8 to 1 mCi/ml) (Easytag; NEN) was then added for labeling for 1 h at 37°C. Cells were washed once with PBS and then chased at 37°C with medium containing nonlabeled methionine and 100 U of aprotinin (Sigma)/ml. At various chase periods, culture medium was collected and washed cells were lysed with PBS containing 0.5% Nonidet P-40, freshly prepared 0.2 M iodoacetamide, and 100 U of aprotinin/ml. Cleared lysates were generated by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge for 10 min.

Extracts of transfected cells were immunoprecipitated with rabbit polyclonal anti-F1 antibody and protein A-Sepharose. The labeled immunoprecipitated proteins were analyzed by SDS-PAGE followed by fluorography.

**Preparation of plasmid DNA for immunization.** The Endofree plasmid preparation kit (Qiagen Ltd., Hilden, Germany) was used for preparation of plasmid DNA stocks. Alternatively, large-scale production of plasmid DNA was performed by the alkali lysis method followed by CsCl gradient centrifugation. The concentration of plasmid DNA was determined by measurement of optical density at 260 nm, and its purity was evaluated by calculation of the $A_{260}/A_{280}$ ratio ($\geq$1.8). DNA size and homogeneity were determined by 1% agarose gel electrophoresis.

For needle injections, purified DNA preparations were solubilized in pyrogen-free saline (Mini-Plasco; B. Braun) to a concentration of 3 mg/ml and were kept frozen in aliquots at −20°C until use. For gene gun vaccination, plasmid stocks were resuspended at 1 mg/ml in distilled pyrogen-free water and stored at −20°C until use.

DNA preparations were examined for the presence of endotoxins by *Limulus* amebocyte lysate testing using a kit from BioWhittaker (Walkersville, Md.). Endotoxin levels in all preparations were found to be lower then 0.05 endotoxin units/ml, which is the detection limit of the kit.

**Bacterial strain used for challenge.** All challenge experiments were performed with the *Y. pestis* Kimberley53 strain. Kimberley53 was obtained by passage of the Kimberley strain (originating from Instituto Oswaldo Cruz, Rio de Janeiro, Brazil [5]) in mice (subcutaneous [s.c.] inoculation and harvesting from spleen).

The $SCLD_{50}$ of this strain in mice is as low as 1 CFU. No difference in virulence was observed when ICR, BALB/c, and DBA/2 mice were tested. Bacteria for challenge were grown for 48 h at 28°C on brain heart infusion agar plates (Difco).

**Immunization and challenge with *Y. pestis*.** The female outbred ICR mice and inbred BALB/c and DBA/2 mice (5 to 6 weeks old) used for vaccination were handled in accordance with the National Institute of Health's guide for the care and use of laboratory animals and the guidelines of the local commission for animal care.

For DNA immunization by needle injections, a protocol of three or four immunizations at 2-week intervals was used. For each immunization, 200 μg of DNA was administered either into the tibialis anterior muscles of the two hind legs or into the tail base dermis.

For gene gun immunization, plasmid DNA was precipitated onto 1-μm-diameter gold particles at a ratio of 2 μg per milligram of gold and loaded onto Gold-Coat tubing as suggested by the manufacturer (Bio-Rad). Polyvinylpyrrolidone (MW, 360,000) was used as an adhesive at a concentration of 0.05 mg/ml. Agarose gel electrophoresis was used to determine the amount of DNA. Vaccination was carried out with three immunizations of 0.5 μg of DNA at 2-week intervals. Gene gun shots were directed into exposed abdominal dermis. At the indicated times, serum was collected in serum separator tubes (Microtainer tubes; Becton Dickinson). Antibody enzyme-linked immunosorbent assay (ELISA) titers were determined as described below. Immunized mice were challenged by s.c. injection of the indicated amounts of *Y. pestis* Kimberley53 strain suspension (0.1 ml). Animals were monitored daily for survival for a period of 3 weeks.

Passive immunization of mice was performed by transfer of serum derived from DNA-immunized mice or from mice immunized with F1 protein (see below). Antiserum aliquots of 1 ml per mouse were injected intraperitoneally (i.p.). Blood was withdrawn from the tail vein 10 h after transfer, and the levels of anti-F1 antibodies in the circulation were determined. Three hours later, the passively immunized mice were challenged with *Y. pestis* as described above.

**Generation of purified F1 antigen and anti-F1 antisera.** F1 antigen was extracted from *Y. pestis* EV76 (5) and purified by the method described by Baker and coworkers (4) to an estimated purity of ~95%, as evaluated by SDS-PAGE (Fig. 1B). To generate anti-F1 antibodies, female ICR mice were vaccinated by

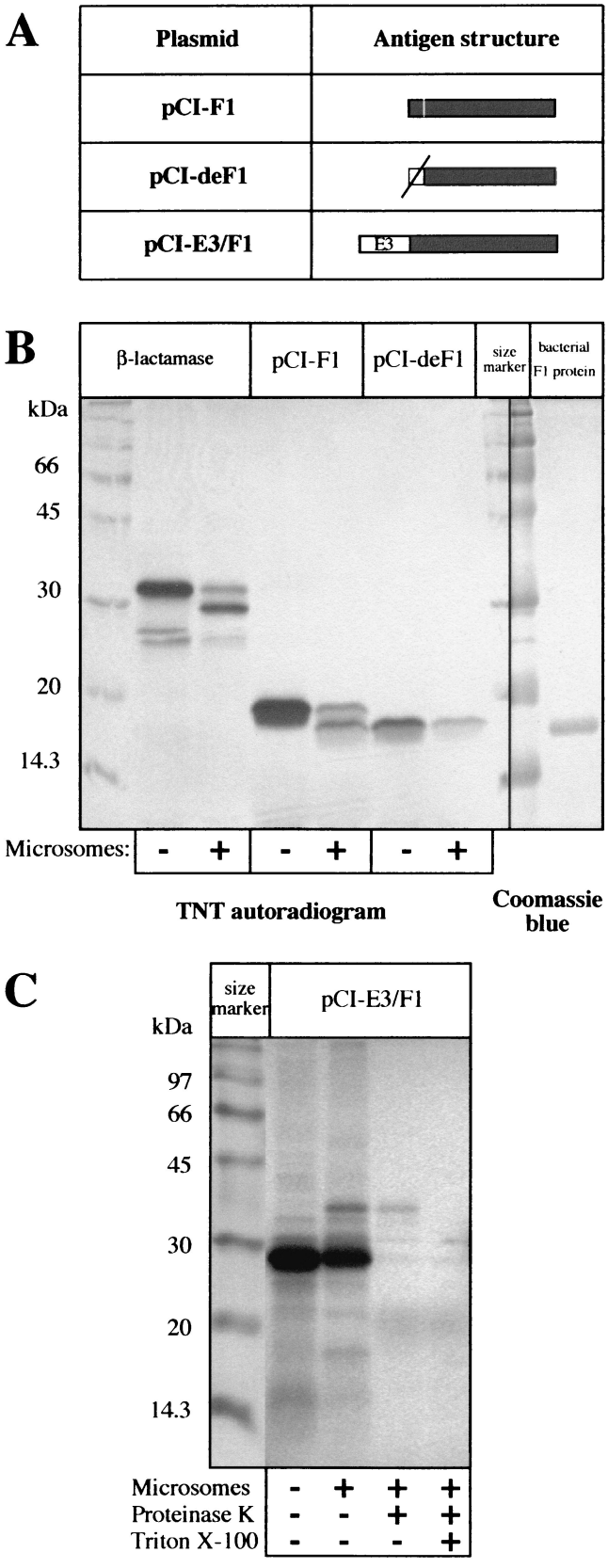


FIG. 1. Generation of vectors for cellular targeting of F1 expression. (A) The three F1 DNA derivatives used in the study are depicted. pCI-F1 expresses the intact bacterial coding sequence of the F1 antigen, pCI-deF1 expresses the putatively mature F1 devoid of the bacterial signal sequence, and pCI-E3/F1 expresses F1 fused to the E3 envelope protein of SFV carrying an eukaryotic signal peptide. (B and C) Radiolabeled, in vitro expression products of pCI-F1, pCI-deF1, and pCI-E3/F1. Polypeptides were generated in a TNT system in the presence or absence of canine microsomes. The β-lactamase cDNA was used as the control for signal cleavage. Purified bacterial F1 was used as the reference for migration of the mature gene product. Prestained, $^{14}$C-labeled polypeptides (Rainbow; Pharmacia) served as MW markers. In panel C, translocation of the E3/F1 hybrid into microsomes was examined by proteinase K treatment in the presence or absence of Triton X-100.

three injections of purified F1 with incomplete Freund's adjuvant (ICFA) at 2-week intervals. Serum was collected 6 weeks postvaccination.

**Antisera and serological tests.** Determinations of mouse anti-F1 immunoglobulin G (IgG) antibody titers were performed by capture ELISA using the purified F1 antigen. Fifty-nanogram aliquots of F1 antigen were added to 96-well microtiter plates coated with rabbit polyclonal anti-F1 antibodies. After incubation for 1 h at 37°C, excess antigen was removed by washing. Twofold dilutions of the examined mouse sera were added to wells for 1 h at 37°C. Alkaline phosphatase-labeled rabbit anti-mouse IgG (1/2,000 dilution; Sigma) was used as the second layer. Titers were determined as reciprocal numbers of the highest serum dilution which displayed values for optical density at 405 nm that were twofold higher than that for control serum. For determination of anti-F1 IgG1 and IgG2a, the serially diluted F1-bound mouse antibody in the microtiter plates (see above) was reacted with ISO-2 (Sigma) anti-mouse antibody isotyping reagents prior to development with alkaline phosphatase rabbit anti-goat conjugates (Sigma).

## RESULTS

**Generation of expression vectors for subcellular targeting of F1 expression.** Three plasmids carrying different cellular localization signals (Fig. 1A) were designed to direct the expression of the *Y. pestis* F1 antigen in eukaryotic cells. pC1-F1 carries the entire intact bacterial F1 coding sequence, including the 21-amino-acid-long putative bacterial signal peptide (16). pC1-deF1 carries the coding sequence of the F1 protein but is devoid of the putative bacterial signal sequence. pCI-E3/F1 codes for the bacterial protein fused to a short viral protein which provides eukaryotic signals for exporting proteins out of the cell. In the third construct, we used the E3 envelope protein of SFV (17), a 66-amino-acid-long peptide involved in the translocation of the 110-kDa viral envelope precursor, resulting in the formation of the viral spike structure located on the outer side of the cell membrane (28).

The TNT system was used for the analysis of these three constructs to allow for protein size verification, antigen authentication, and assessment of putative processing in the cell by monitoring translocation into canine microsomes. All three constructs expressed the F1 derivatives and were recognizable by specific antibodies (Fig. 1B and C). The full-length F1 protein expressed by pCI-F1 exhibits a MW of 17,000 to 18,000, which is as expected from its amino acid composition (16). Truncation of its putative signal sequence (pCI-deF1) resulted in a shorter polypeptide (~15 kDa), which comigrated with the native F1 isolated from *Y. pestis* cultures (Fig. 1B). The size of the E3/F1 hybrid was ~28 kDa, as expected from its putative sequence (Fig. 1C).

Addition of canine microsomes to the translation system had no effect on deF1 but resulted in the formation of a smaller polypeptide in the case of the full-length F1. The newly formed cleavage product comigrated with the translation product of

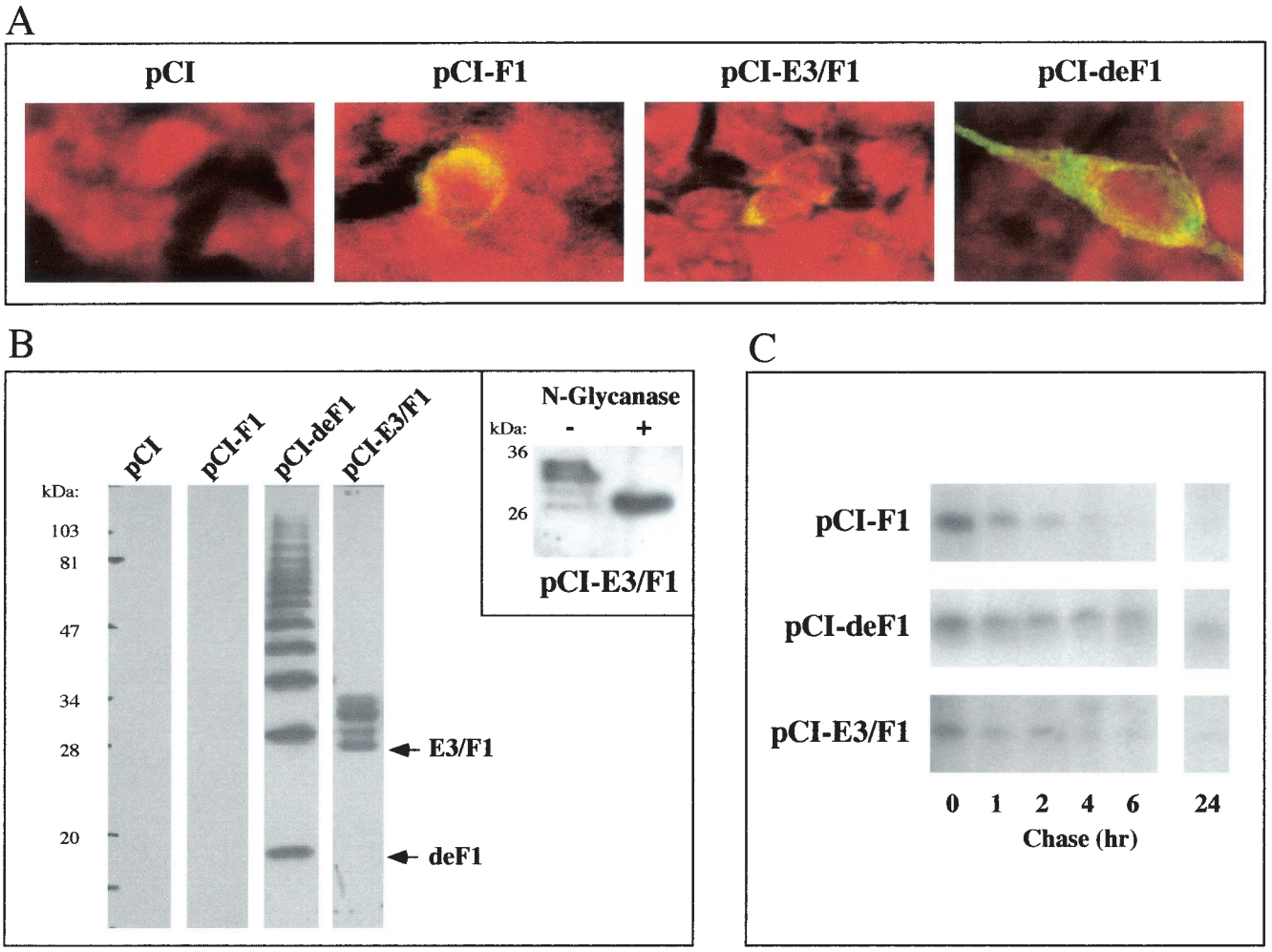


FIG. 2. The fate of F1 derivatives expressed in transfected HEK293 cells. Cells were cultivated 24 h posttransfection and then submitted to the various procedures as described in Materials and Methods. Transfection by pCI was used as the negative control. Expression of F1, deF1, and E3/F1 in transiently transfected cells was monitored by immunofluorescence staining of cells (A), by immunoblotting of cell extracts (B), and by immunoprecipitation of metabolically labeled cellular polypeptides (C) with antibodies to bacterial F1. The inset in panel B depicts the effect of *N*-glycanase treatment on the SDS-PAGE mobility of E3/F1.

the deF1 DNA as well as with the native bacterial F1 (Fig. 1B). This suggests that the putative signal peptide of F1 is functional and is actually recognized by a eukaryotic translocation system.

Addition of microsomes to the TNT system expressing E3/F1 hybrid DNA resulted in the appearance of a new, slow-migrating product (Fig. 1C). The new band represented only a small fraction of the newly translated E3/F1, yet the signal was distinct and appeared only upon microsome addition (TNT experiments with the native viral envelope proteins yielded similar intensities [data not shown]). This larger polypeptide appeared to reside within the lumen of the microsome, as it was resistant to proteinase K treatment of intact microsomes yet was sensitive to proteolysis upon disruption of the microsomes by detergent. These observations suggest that E3/F1 is capable of translocation into the membranal fraction and apparently undergoes glycosylation (see below), as manifested by the MW shift. It should be noted that when the pCI-F1 and pCI-deF1 translation products were subjected to the same treatment (proteinase K digestion of the microsome-supplemented TNT product), no indication of translocation into the microsomal lumen could be obtained (data not shown).

**Processing of F1 derivatives in transfected cells.** To determine the fate of the various F1 derivatives in the cell, HEK293 cells were transfected with the plasmids coding for full-length F1 (pCI-F1), mature F1 (pCI-deF1), or F1 linked to the SFV secretion system (pCI-E3/F1). All three forms were expressed in the human cell line, as suggested by staining with fluorescein-labeled antibodies (Fig. 2A); however, the staining pattern did not appear to be the same. Cells expressing deF1 exhibited intense fluorescence dispersed throughout the cell, characteristic of cytosolic staining. On the other hand, in cells expressing the other two forms, staining was less intense and appeared to be centered around the nucleus, which could indicate localization in the cisternae of the secretory pathway.

Processing of the three F1 derivatives in the cells was evaluated by examining cell-associated F1 at steady state. To this end, cells expressing the three F1 constructs were lysed and

their protein content was analyzed by Western blotting (Fig. 2B). This analysis revealed clear differences in the behavior of the three F1 derivatives. In cells expressing deF1, the expected ~15-kDa polypeptide was accompanied by a ladder of bands of 30, 45, and 60 kDa and greater (the ratio of band intensities varied between different preparations). This pattern suggests formation of aggregates of increasing size and is in agreement with the ability of mature F1 produced by *Y. pestis* to form aggregates upon capsule formation (35). Cells expressing the E3/F1 hybrid also exhibited polymorphic F1 polypeptides, but this polymorphism was clearly distinct from that of deF1. Here, the expected 28-kDa band was accompanied by a ladder of diffuse bands closer in size (Fig. 2B), a pattern which is characteristic of glycosylation-derived heterogeneity. Indeed, treatment of this cell extract with *N*-glycanase resulted in the disappearance of the larger bands (Fig. 2B, inset). In cells expressing full-length F1, the polypeptide could not be identified in Western blots under the same assay conditions (Fig. 2B); nevertheless, a faint band of ~17.5 kDa was detected when the concentration of anti-F1 antibody used for blot development was increased by 10-fold (data not shown). The small amount of full-length F1 detected by Western blotting appears to be inconsistent with the intensive immunostaining observed in cells expressing this derivative and could result from generation of degraded low-MW products, as is indeed suggested by the rapid elimination of F1 from transfected cells (see below).

To further evaluate the biogenesis of the various F1 configurations, transfected HEK293 cells were metabolically labeled with [$^{35}$S]methionine for 1 h followed by addition of cold methionine. The presence of F1 antigen in cells was monitored at various times by immunoprecipitation (Fig. 2C). All three F1 derivatives could be detected in cells following 1-h pulse with [$^{35}$S]methionine: F1 and deF1 appeared as bands of ~17.5 and ~15 kDa, respectively, and E3/F1 appeared as a ~33-kDa band, suggesting that only the last polypeptide underwent glycosylation. Of the three forms, the full-length F1 was cleared from cells most quickly, with an approximate half-life of 1 h. The band intensity of the other two forms decreased more gradually, and faint bands could still be detected in cell extracts after 24 h (Fig. 2C). In the case of F1 and deF1, no labeled polypeptide was detected in the HEK293 cell medium. As for E3/F1, attempts to detect labeled F1-polypeptide were unsuccessful since in mock-transfected HEK293 cells anti-F1 antibodies precipitated a secreted band with an electrophoretic mobility similar to that of E3/F1.

Taken together, these observations indicate a different cellular fate for the three F1 forms. Linkage to the SFV E3 polypeptide appears to direct the hybrid F1 polypeptide to the cisternae of the secretory pathway, whereas deliberate omission of the signal peptide ensures retention of the newly formed deF1 molecules in the cytoplasm for a prolonged period of time. The cellular fate of the full-length F1 polypeptide carrying the nonmodified bacterial signal appears to be different from that of the other two forms, and it is prone to accelerated elimination from the cell by an as-yet-unknown mechanism.

**Antibody production in mice immunized with F1 plasmid vaccines.** To evaluate the potential of the various F1 plasmids as DNA vaccines, purified preparations of the plasmid DNAs coding for full-length bacterial F1, deF1, and E3/F1 chimera were injected into ICR mice by using the vector pCI as a negative control. Anti-F1 titers were determined 2 weeks following the last immunization. As shown in Fig. 3, plasmid pCI-F1, coding for the full-length F1 form, was found to be a poor anti-F1 IgG inducer (geometric mean titer [GMT] = 120). In contrast, the plasmids harboring the two genetically manipulated F1 derivatives, deF1 and E3/F1 hybrid, were found to induce substantial IgG titers, with GMTs of 2,700 (geometric standard deviation [GSD], −2.5) and 900 (GSD, −1.8), respectively. This significant difference ($P < 0.01$; $t$ test) in response between deF1 and E3/F1 DNAs was found to be reproducible in three independent experiments. Furthermore, deF1 proved to be superior to E3/F1 when the two DNA vaccines were compared for their ability to prime antibody induction when a booster of alum-absorbed F1 polypeptide was injected 14 days following three DNA injections. A GMT of 400,000 was achieved 2 weeks after the booster injection for the deF1-primed mice, compared with a GMT of 80,000 achieved with E3/F1 DNA.

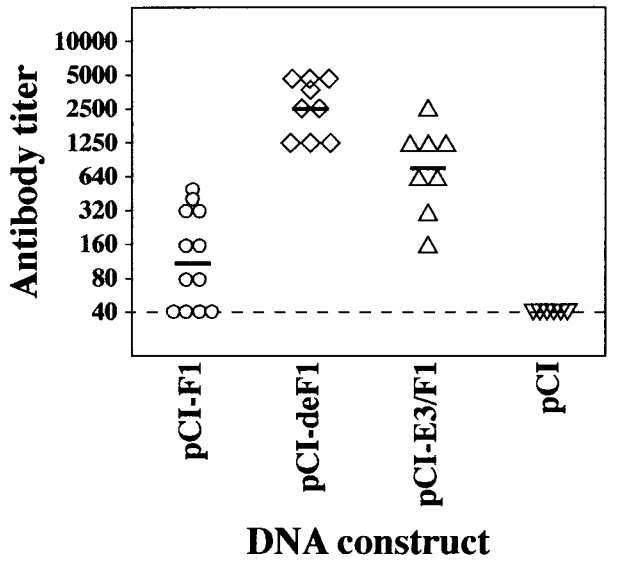


FIG. 3. Induction of antibodies to F1 by the various DNA vaccine derivatives. pC1-F1, pC1-deF1, and pC1-E3/F1 DNAs were injected i.m. into the hind legs of 6-week-old female ICR mice. Three doses of 200 μg each were injected at weeks 0, 2, and 4. Anti-F1 IgG titers were determined in individual animals by ELISA 2 weeks following the third injection. Individual titers in each group are presented, and GMTs are indicated with thick horizontal lines. The ELISA assay baseline is indicated with a dotted line.

To further evaluate the potential of deF1 DNA as a vaccine, we compared its ability to induce antibody production in three different mouse strains: outbred ICR mice and inbred BALB/c and DBA/2 mice. Two modes of DNA administration, intramuscular (i.m.) and intradermal (i.d.), were examined. The results presented in Table1 indicate that anti-F1 IgG induction was dependent neither on the mice strain nor on the route of vaccination. All animal strains immunized with deF1 DNA produced anti-F1 IgG at comparable titers. In inbred mice, no substantial difference was observed between animals immunized i.m and those immunized i.d. In outbred mice, i.m. im-

TABLE 1. Effect of mouse strain and mode of deF1 DNA vaccination on antibody induction

| Strain | Mode of immunization | Anti-F1 IgG GMT (GSD) after[a]: | | Survival (no. alive/total)[b] |
|---|---|---|---|---|
| | | Third injection | Fourth injection | |
| ICR | i.m. | 4,800 (1.3) | 19,000 (2.3) | 5/5 |
| | i.d. | 1,690 (2.2) | 7,130 (2.0) | 5/5 |
| | Control[c] | <50 | <50 | 0/5 |
| BALB/c | i.m. | 1,310 (2.2) | 6,620 (2.4) | 5/5 |
| | i.d. | 1,900 (1.9) | 9,100 (2.4) | 4/4 |
| | Control | <50 | <50 | 0/5 |
| DBA/2 | i.m | 2,400 (2.2) | 4,160 (1.9) | 5/5 |
| | i.d. | 2,300 (1.6) | 6,450 (2.0) | 4/5 |
| | Control | <50 | <50 | 0/4 |

[a] Titers were determined 10 days following the third or fourth injection.
[b] Animals were challenged s.c. by 50 CFU of *Y. pestis* strain Kimberly53 3 weeks after the fourth injection.
[c] pCI DNA-vaccinated mice (i.m., three injections).

munization appears to provide some advantage. It should be noted that in all mouse groups examined a pronounced increase in antibody titer was observed when the number of DNA injections was increased from three to four.

For further evaluation of the potential of deF1 DNA as a vaccine, immunizations by gene gun bombardment and needle injections were compared. Gene gun immunization was found to be preferable, both by the level of antibody titers and the amount of DNA vaccine needed for vaccination. A very low dose of plasmid DNA (0.5 μg) per gene gun shot was sufficient to induce a very high titer of anti-F1 antibodies, compared with the 200 μg per injection required for needle immunization. Moreover, after three gene gun injections, the antibody titers were as high as 55,000, compared with ~8,000 and ~19,000 after three and four needle vaccinations, respectively (Fig. 4A). It should be noted that high levels of antibody titers were maintained for a long period of time postvaccination. These findings were consistent and were observed in three independent experiments. A moderate decrease in titers in needle-vaccinated animals as well as in gene gun-vaccinated animals was observed 18 weeks postvaccination (Table 2).

Subtyping of antibodies induced by F1 DNA vaccination revealed the expected difference between needle and gene gun vaccinations (15). Needle vaccination was characterized by higher titers of IgG2a than of IgG1. This ratio was inverted in gene gun vaccination, where IgG1 antibodies were prominent. In this respect, gene gun DNA immunization resembles immunization with ICFA-formulated F1 antigen (Fig. 4B).

**Protective immunity induced by F1 DNA vaccination.** The ability of DNA coding for deF1 to confer protective immunity was examined by challenging the vaccinated animals with increasing doses of *Y. pestis*. To this end, we used s.c. inoculation with the virulent Kimberley53 strain. This protocol mimics the natural infection mode via flea bites and provides the most sensitive mode of infection in mice (50% lethal dose [$LD_{50}$] equaling 1 CFU). In the first set of experiments, the i.m. and i.d. vaccinated inbred and outbred mice (Table 1) were challenged with 50 $LD_{50}$s of *Y. pestis* 3 weeks postimmunization. Practically full protection was achieved independent of the

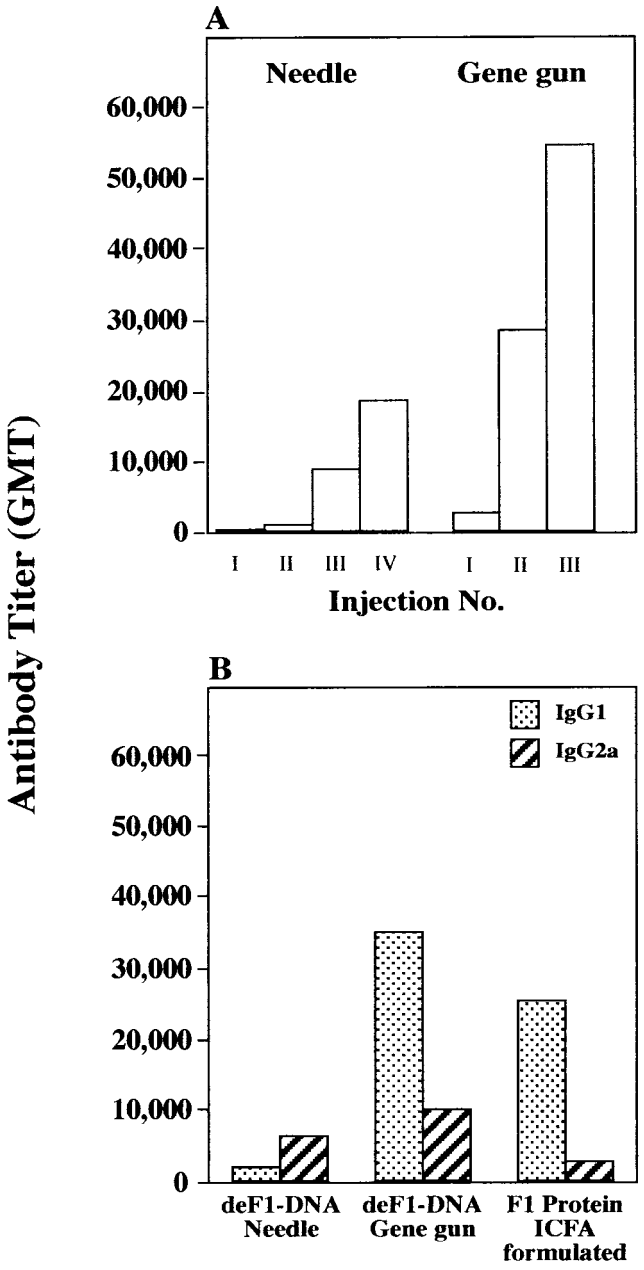


FIG. 4. Comparison of anti-F1 antibody induction by needle and gene gun immunization. Mice (10 per group) were immunized either by four i.m. needle immunizations (200 μg of DNA per dose) or by three gene gun bombardments (0.5 μg of DNA per dose) as described in Materials and Methods. Antisera were collected 2 weeks after each vaccination. Shown are the development of antibody titers (A) and antibody subtypes (B). Subtyping was performed after the last vaccination. For comparison, IgG subtypes in a hyperimmune antiserum obtained by vaccination with bacterial F1 polypeptide formulated in ICFA (see Materials and Methods) were determined as well.

mouse strain or the mode of vaccination (Table 1). In total, 38 of 39 animals survived challenge. The GMTs of specific anti-F1 antibodies in the various groups ranged from 4,000 to 19,000.

After defining the effective modes of vaccination, protection against higher challenge doses for a prolonged period of time

TABLE 2. Effective protection by deF1 DNA immunization

| Mode of injection | Anti-F1 IgG GMT (GSD)[a] | | Challenge dose (CFU)[b] | Survival (no. alive/total) |
|---|---|---|---|---|
| | 3 wk p.i. | 18 wk p.i. | | |
| Needle[c] | 9,600 (1.6) | 2,500 (2.3) | 400 | 5/5 |
| Gene gun[d] | 50,000 (2.0) | 20,000 (1.6) | 400 | 5/5 |
| Gene gun[d] | 55,000 (1.7) | 26,000 (2.0) | 4,000 | 10/10 |
| Control[e] | <40 | <40 | 400 | 0/10 |

[a] Titers were determined 10 days following the third or fourth injection. p.i., postimmunization.
[b] 1 CFU = 1 $SCLD_{50}$.
[c] Four injections of 200 μg of DNA each at 2-week intervals.
[d] Three shots of 0.5 μg of DNA each.
[e] Mice were vaccinated with pCI DNA by gene gun.

was examined (Table 2). Mice vaccinated by four i.m. needle injections (GMT = 2,500) or three gene gun shots (GMT = 20,000) of pC1-deF1 were challenged 18 weeks postvaccination with 400 $LD_{50}$s, and all survived challenge. To further evaluate the protective potential of the deF1 DNA vaccine, gene gun-immunized mice (GMT = 26,000) were challenged with 4,000 $LD_{50}$s, and all 10 animals in the assay group survived this high dose of virulent *Y. pestis*.

To assess the role of antibodies in the protective immunity conferred by DNA vaccination, passive transfer experiments were conducted. To this end, high-titer serum pools (titers of ~30,000) from mice immunized with either deF1 DNA or F1 polypeptide formulated in ICFA were generated and administered i.p. to mice. The actual titers in the circulation of the recipient animals were 1,000 to 8,000 at 10 h after transfer. In the first set of experiments, mice carrying antisera to the F1 polypeptide vaccine were challenged s.c. with 40 and 400 $LD_{50}$s of *Y. pestis* (Fig. 5A and B). The passively transferred anti-polypeptide serum conferred effective protection against 40 $LD_{50}$s (Fig. 5A) and was partially effective against 400 $LD_{50}$s (Fig. 5B). At the lower challenge dose, four of five animals were protected. At the higher challenge dose, two mice were protected, while the deaths of the other three were delayed by 2 days; all control animals died within 6 to 7 days. In the next experiment, recipient mice carrying antisera to the deF1 DNA vaccine were challenged s.c. with 100 $LD_{50}$s (Fig. 5C). In this case, three of five animals were protected and the deaths of the other two were delayed by 5 days compared with those of naive animals. These results suggest that antiserum induced by the DNA vaccine is as effective as that induced by the F1 polypeptide in conferring protection against lethal doses of *Y. pestis*.

## DISCUSSION

F1 in its native or recombinant form is a potent immunogen known to induce high titers of circulating IgG as well as protection against high challenge doses of virulent *Y. pestis* (3, 24, 29). Nevertheless, when a DNA vaccine based on nonmodified F1 gene was injected into outbred mice, a poor antibody response was detected (Fig. 3). This finding is in good agreement with observations in a previous study (9), where a similar DNA construct elicited a very poor immune response. The discrepancy between these results and the notable immunogenicity of the F1 polypeptide could originate from inadequate presentation of the DNA vaccine gene product to the immune system. The goal of this study was to find the optimal DNA vaccine for induction of a strong humoral response that confers protection against *Y. pestis* infection.

The F1 gene codes for a polypeptide of 17.5 kDa that appears to possess a secretion signal at the N terminus (16). While the functionality of this signal was not demonstrated directly, recent mass spectrometric analyses have indicated that the actual MW of F1 is 15.5 kDa (31), suggesting that the first 21 amino acids of the polypeptide are cleaved off upon maturation. Moreover, when analyzed against a eukaryotic database by using the Signal algorithm, the putative bacterial signal of F1 was found to exhibit features of a eukaryotic signal as well. Indeed, treatment of the full-length F1 in vitro translation product with canine microsomes resulted in the formation of a shorter polypeptide, similar in size to the mature bacterial F1 (Fig. 1B).

The presence of a functional secretion signal could play a part in determining the low level of response to F1 DNA vaccination. To examine this possibility, we generated two modified F1 DNA constructs, one coding for a truncated F1 (deF1) devoid of the 21 amino acid signal and the other in which an authentic eukaryotic secretion signal was introduced through fusion of F1 to the SFV E3 polypeptide. Both modified constructs proved to be superior to native F1 DNA in their ability to induce antibody response (Fig. 3). The expressions of these two engineered genes appear to be targeted to different cellular compartments. The results of TNT assays as well as cell transfection experiments suggested that the E3/F1 hybrid is translocated to the vesicles of the secretion pathway, where it appears as a heterogeneously glycosylated form (Fig. 1C and 2B), consistent with the presence of three putative glycosylation signals on the hybrid. On the other hand, deF1 remains localized in the cytosol, where it is able to form discrete multimers (Fig. 2B), resembling the native bacterial capsid product. These two F1 derivatives appear to be gradually eliminated from transfected cells. In the case of deF1, this is probably the result of cytosolic degradation, whereas in the case of E3/F1, this can reflect either export from the cell or intracellular degradation of the translocated polypeptide. Our attempts to demonstrate export of E3/F1 in HEK293 cells failed, yet with COS cells, we were able to detect small quantities of glycosylated E3/F1 in cell medium (data not shown).

In contrast to the relatively slow elimination of deF1 and E3/F1 from the cell, nonmodified F1 is rapidly cleared from transfected 293 cells. The half-life of labeled nontruncated F1 appears to be about 1 h, and the polypeptide is hardly detectable in the cell at steady state (Fig. 2B and C). It should be noted that the cellular fate of F1 expressed from the full-length DNA could not be fully resolved. While cleavage of its signal by microsomes (Fig. 1B) and the uneven staining in transfected cells (Fig. 2A) could suggest translocation into the secretory vesicles, the inability to demonstrate glycosylation (F1 carries one putative N-glycosylation signal) or export from the cell precludes a clear-cut cellular assignment.

Vaccination with deF1 as well as with E3/F1 DNA resulted in titers which were at least 1 order of magnitude higher than those induced by full-length F1 DNA (Fig. 3). While possible differences in the translation rates of the various F1 derivatives cannot be precluded, the pronounced differences in cellular processing of the various gene products strongly suggest that

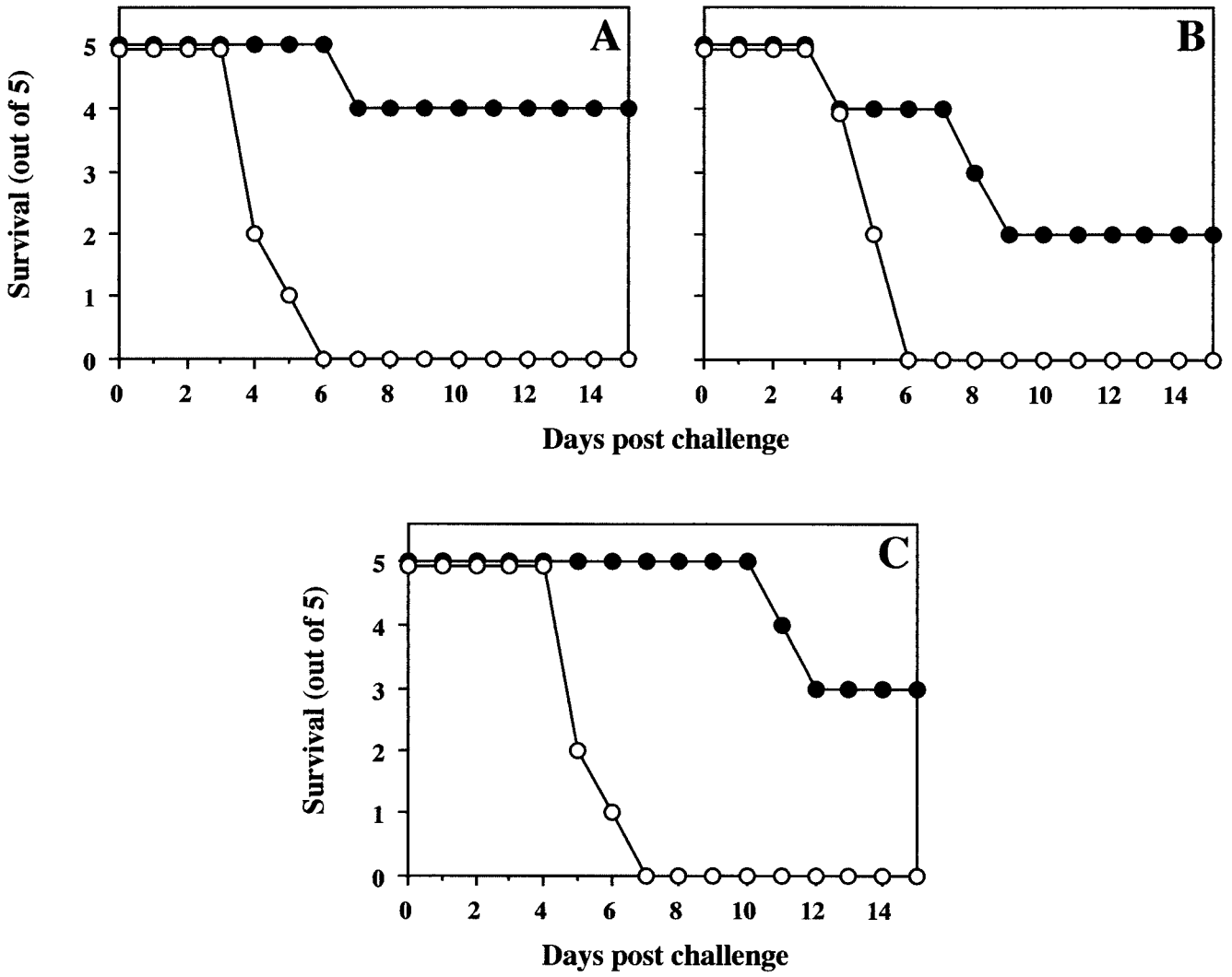


FIG. 5. Conferring protection to *Y. pestis* by passive transfer of anti-F1 antibodies. Pooled mouse antisera generated by F1 polypeptide immunization (A and B) or deF1 DNA vaccination (C) were transferred to ICR mice as described in Materials and Methods. F1 polypeptide was formulated in ICFA, and deF1 DNA was administered by gene gun (Fig. 4). Animals were challenged 12 h after serum transfer by s.c. injection of 40 (A), 400 (B), or 100 (C) $LD_{50}$s of virulent *Y. pestis*. Control groups of five animals consisted of nonimmunized mice receiving the same challenge doses. Survival was followed for 15 days (solid circles represent passively vaccinated animals and open circles represent controls).

this phenomenon is responsible for the difference in their potentials as vaccines.

The actual process of antigen presentation upon DNA vaccination is still under debate (10, 18), and the processing pathways of the F1 derivatives in antigen-presenting cells are not necessarily similar to those in transfected HEK293 cells. It is difficult, therefore, to draw a correlation between the residence time of F1 antigens in HEK293 cells and their ability to induce antibody response upon vaccination. Yet, rapid elimination of full-length F1 from cells may also occur in vivo and thus preclude its proper presentation to the MHC mediators, while gradual rates of deF1 and E3/F1 degradations would allow a more effective presentation. Interestingly, the lack of a major difference in elimination rates and the similar immunogenicity between F1 derivatives targeted to the cytosol (deF1) and endoplasmic reticulum (E3/F1) may imply that both forms undergo the same process of fragmentation prior to presentation. This, in turn, may possibly implicate the involvement of retrograde transport from the endoplasmic reticulum to the cytosol (the ERAD pathway [25]) in E3/F1 degradation.

Further examination of the potential of DNA vaccination against plague involved deF1 DNA. The deF1 DNA product formed in the cytosol of the eukaryotic cell retains its ability to aggregate, resembling the naturally formed bacterial protein (35). This observation is also consistent with the predicted beta-sheet secondary structure of the protein (16) and suggests that the newly expressed deF1 maintains a native functional configuration in the cell. The deF1 DNA elicited antibodies in BALB/c and DBA/2 mice as efficiently as in outbred mice (Table 1). This is in contrast to previous observations with nonmanipulated F1 (9), where sensitivity to the mouse genetic background was observed. Moreover, all vaccinated mice, regardless of strain, were resistant to a challenge by 50 $LD_{50}$s of virulent *Y. pestis* (Table 1). In order to induce higher re-

sponses, which would presumably confer resistance to higher doses of bacteria, the gene gun bombardment approach was examined. Indeed, three such immunizations resulted in titers as high as 50,000, similar to those displayed when we employed a three-dose vaccination regimen with F1 polypeptide formulated with alum or ICFA (data not shown). Such antibody titers for bacterial DNA vaccination are exceptionally high and are comparable to those usually achieved with a regimen which involves priming by DNA and boosting by free protein (9). The antibody response to gene gun bombardment with deF1 DNA was characterized by IgG1 predominance (Fig. 4), which is typical of this mode of DNA administration (15) and is indicative of an ongoing Th2 response (30). It is interesting that Th2 response has been observed in many other cases in which DNA vaccines have been administered by gene gun and where expression is most probably localized in the cytosol (6, 11). Thus, the assumption that cytosolic expression of exogenous DNA is primarily associated with activation of the nonhumoral arm of the immune response is not necessarily valid in the case of gene gun DNA vaccination.

Vaccination by gene gun conferred resistance to *Y. pestis* challenges as high as 4,000 $LD_{50}$s. The essential role of antibody response in the protective immunity conferred by F1 protein-based vaccine (3, 14) and in particular the observed correlation between anti-F1 IgG1 and resistance to challenge (40), suggest that the protective value of deF1 DNA is due to its ability to induce elevated antibody response to F1. Indeed, when antibodies derived from the DNA-vaccinated mice were transferred to naive mice, significant protection against a challenge of 100 $LD_{50}$s was observed. This protection was as efficient as that conferred upon passive transfer from F1 polypeptide-vaccinated mice (Fig. 5). This observation indicates that humoral immunity plays a central role in the protection conferred by deF1 DNA against plague but does not preclude the involvement of cellular immunity as well.

Taken together, the results of this study demonstrate that DNA-based vaccines can confer effective protection not only against viral pathogens and intracellular parasites (listed in reference 26) but also against essentially extracellular bacteria as well. A rather simple protocol, without the need to resort to complex regimens involving DNA priming and boosting with protein (9, 41), was found to be effective against one of the most infectious pathogens. This was achieved by the proper tailoring of the DNA through removal of the bacterial secretion signal, thus directing cytosolic expression which is not prone to rapid elimination. Though DNA vaccine development does not seem to abide by general rules, the approach used here may be beneficial in overcoming difficulties encountered in developing DNA vaccines against other pathogenic bacteria.

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## REFERENCES

1. **Abramov, V. M., A. M. Vasiliev, R. N. Vasilienko, N. L. Kulokova, I. V. Kosarev, V. S. Khlebnikov, A. T. Ishchenko, S. MacIntyre, J. L. Gillespie, R. Kurana, T. Korpela, A. L. Fink, and V. N. Uversky.** 2001. Structural and functional similarity between *Y. pestis* capsular protein Caf1 and human interleukin-1β. Biochemistry **40:**6076–6084.
2. **Anderson, G. W., D. G. Heath, C. R. Bolt, S. L. Welkos, and A. M. Friedlander.** 1998. Short and long-term efficacy of single-dose subunit vaccines against *Yersinia pestis* in mice. Am. J. Trop. Med. Hyg. **58:**793–799.
3. **Andrews, G. P., D. G. Heath, G. W. Anderson, Jr., S. L. Welkos, and A. M. Friedlander.** 1996. Fraction 1 capsular antigen purification from *Yersinia pestis* CO92 and from an *E. coli* recombinant strain and efficacy against lethal plague challenge. Infect. Immun. **64:**2180–2187.
4. **Baker, E. E., H. Sommer., L. E. Foster, E. Meyer, and K. F. Meyer.** 1952. Studies on immunization against plague. I. Isolation and characterization of the soluble antigen of *Pasteurella pestis*. J. Immunol. **68:**131–145.
5. **Ben-Gurion, R., and I. Hertman.** 1958. Bacteriocin-like material produced by *Pasteurella pestis*. J. Gen. Microbiol. **19:**289–297.
6. **Bennett, A. M., R. J. Phillpotts, S. D. Perkins, S. C. Jacobs, and E. D. Williamson.** 1999. Gene gun mediated vaccination is superior to manual delivery for immunization with DNA vaccines expressing protective antigens from *Yersinia pestis* or Venezuelan Equine Encephalitis virus. Vaccine **18:** 588–596.
7. **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
8. **Boyle, J. S., C. Koniaras, and A. M. Lew.** 1997. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. Int. Immunol. **9:**1897–1906.
9. **Brandler, P., K. U. Sakh, D. Heath, A. Friedlander, and R. G. Urlich.** 1998. Weak anamnestic responses of inbred mice to *Yersinia* genetic vaccine are overcome by boosting with F1 polypeptide while outbred mice remain nonresponsive. J. Immunol. **161:**4195–4200.
10. **Cohen, A. D., J. D. Boyer, and D. B. Weiner.** 1998. Modulating the immune response to genetic immunization. FASEB J. **12:**1611–1626.
11. **Davis, H. L., and M. J. McCluskie.** 1999. DNA vaccines for viral diseases. Microbes Infect. **1**:7–21.
12. **Davis, K. M., D. L. Fritz, L. M. Pitt, S. L. Welkos, P. L. Worsham, and A. M. Friedlander.** 1996. Pathology of experimental pneumonic plague produced by fraction 1-positive and fraction 1-negative *Yersinia pestis* in African green monkeys. Arch. Pathol. Lab. Med. **120:**156–163.
13. **Du, Y., R. Rosqvist, and A. Forsberg.** 2002. Role of fraction 1 antigen of *Yersinia pestis* in inhibition of phagocytosis. Infect. Immun. **70:**1453–1460.
14. **Elvin, S. J., and E. D. Williamson.** 2000. The F1 and V subunit vaccine protects against plague in the absence of IL-4 driven immune responses. Microb. Pathog. **29:**223–230.
15. **Feltquate, D. M., S. Heaney, R. G. Webster, and H. L. Robinson.** 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J. Immunol. **158:**2278–2284.
16. **Galyov, E. E., O. Y. Smirnov, A. V. Karlishev, K. I. Volkovoy, A. I. Denesyuk, I. V. Nazimov, K. S. Rubstov, V. M. Abramov, S. M. Dalvadyanz, and V. P. Zav'yalov.** 1990. Nucleotide sequence of *Yersinia pestis* gene encoding F1 antigen and the primary structure of the protein. FEBS Lett. **277:**230–232.
17. **Garoff, H., C. Kondor-Koch, R. Petterson, and B. Burke.** 1983. Expression of Semliki Forest virus proteins from cloned cDNA. J. Cell Biol. **97:**652–658.
18. **Gregersen, J. P.** 2001. DNA vaccines. Naturwissenschaften **88:**504–513.
19. **Grosfeld, H., B. Velan, M. Leitner, S. Lustig, B.-E. Lachmi, and A. Shafferman.** 1989. Semliki Forest virus E2 envelope epitopes induce a nonneutralizing humoral response which protects mice against lethal challenge. J. Virol. **63:**3416–3422.
20. **Gurunathan, S., D. M. Klinman, and R. A. Seder.** 2000. DNA vaccines: immunology, application, and optimization. Annu. Rev. Immunol. **18:**927–974.
21. **Kerem, A., C. Kronman, S. Bar-Nun, A. Shafferman, and B. Velan.** 1993. Interrelations between assembly and secretion of recombinant human acetylcholinesterase. J. Biol. Chem. **268:**180–184.
22. **Marshall, J. D., P. J. Bartelloni, D. C. Cavanaugh, P. J. Kadull, and K. F. Meyer.** 1974. Plague immunization. II. Relation to adverse clinical reactions to multiple immunizations with killed vaccine. J. Infect. Dis. **129:**S19–S25.
23. **Meyer, K. F.** 1970. Effectiveness of live or killed plague vaccines in man. Bull. W. H. O. **42:**653–666.
24. **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
25. **Plemper, R. K., and D. E. Wolf.** 1999. Retrograde protein translocation: ERADication of secretory proteins in health and disease. Trends Biochem. Sci. **24:**266–270.
26. **Reyes, A., and H. C. J. Ertl.** 2001. DNA vaccines. Curr. Mol. Med. **1:**217–243.
27. **Saikh, K. U., J. Sesno, P. Brandler, and R. G. Ulrich.** 1998. Are DNA-based vaccines useful for protection against secreted bacterial toxins? Tetanus toxin test case. Vaccine **16:**1029–1038.
28. **Schlesinger, M. J., and S. Schlesinger (ed.).** 1986. Formation and assembly of alphavirus glycoproteins, p. 121–148. *In* The Togaviridae and Flaviviridae. Plenum Press, New York, N.Y.
29. **Simpson, W. J., R. E. Thomas, and T. G. Schwan.** 1990. Recombinant capsular antigen from *Yersinia pestis* induces a protective antibody response in BALB/c mice. Am. J. Trop. Med. Hyg. **43:**389–396.
30. **Snapper, C. M., F. D. Finkelman, and W. E. Paul.** 1988. Differential regulation of IgG1 and IgE by synthesis of interleukin 4. J. Exp. Med. **167:**183–197.
31. **Tito, M. A., J. Miller, K. F. Griffin, E. D. Williamson, R. W. Titball, and C. V.**

**Robinson.** 2001. Macromolecular organization of the *Yersinia pestis* capsular F1 antigen: insight from time-of-flight mass spectrometry. Protein Sci. **10:** 2408–2413.

32. **Torres, C. A. T., K. Yang, F. Mustafa, and H. R. Robinson.** 1999. DNA immunization: effect of secretion of DNA-expressed hemagglutinins on antibody responses. Vaccine **18:**805–814.
33. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felger, V. J. Dwarki, S. H. Gromkoowski, R. R. Deck, C. M. DeWitt, A. Freidman, et al.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745–1749.
34. **Velan, B., H. Grosfeld, C. Kronman, Y. Gozes, A. Lazar, Y. Flashner, D. Marcus, S. Cohen, and A. Shafferman.** 1991. The effect of elimination of intrasubunit disulfide bonds on the activity, assembly, and secretion of recombinant human acetylcholinesterase. J. Biol. Chem. **266:**23977–23984.
35. **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.
36. **Weiss, R. W., J. Durnberger, S. Mostbock, S. Scheiblhofer, A. Hartl, M. Breitenbach, P. Strasser, F. Dorner, I. Livey, B. Crowe, and J. Thalhamer.** 1999. Improvement of the immune response against plasmid DNA encoding OspC of *Borrelia* by an ER-targeting leader sequence. Vaccine **18:**815–824.
37. **Welkos, S. L., K. M. Davis, L. M. Pitt, P. L. Worsham, and A. M. Friedlander.** 1995. Studies on the contribution of the F1-capsule-associated plasmid pFra to the virulence of *Yersinia pestis.* Contrib. Microbiol. Immunol. **13:**299–305.
38. **Williamson, E. D., M. E. Stephen, K. F. Griffin, M. Green, P. Russel, S. E. C. Leary, P. C. F. Oyston, T. Easterbrook, K. M. Reddin, A. Robinson, and R. W. Titball.** 1995. A new improved sub-unit vaccine for plague: the basis of protection. FEMS Immunol. Med. Microbiol. **12:**223–230.
39. **Williamson, E. D., S. M. Eley, A. J. Stagg, M. Green, P. Russell, and R. W. Titball.** 1997. A sub-unit vaccine elicits IgG in serum, spleen cell culture and bronchial washing and protects immunized animals against pneumonic plague. Vaccine **15:**1079–1084.
40. **Williamson, E. D., P. M. Vesey, K. J. Gillhespy, S. M. Eley, M. Green, and R. W. Titball.** 1999. An IgG1 titer to the F1 and V antigens correlates with protection against plague in the mouse model. Clin. Exp. Immunol. **116:**107–114.
41. **Williamson, E. D., A. M. Bennett, S. D. Perkins, R. J. Beedham, J. Miller, and L. W. J. Baillie.** 2002. Co-immunization with a plasmid DNA cocktail primes mice against anthrax and plague. Vaccine **20:**2933–2941.

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