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Effective Plague Vaccination via Oral Delivery of Plant Cells Expressing F1-V Antigens in Chloroplasts▽

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The chloroplast bioreactor is an alternative to fermentation-based systems for production of vaccine antigens and biopharmaceuticals. We report here expression of the plague F1-V fusion antigen in chloroplasts. Site-specific transgene integration and homoplasmy were confirmed by PCR and Southern blotting. Mature leaves showed the highest level of transgene expression on the third day of continuous illumination, with a maximum level of 14.8% of the total soluble protein. Swiss Webster mice were primed with adjuvant-containing subcutaneous (s.c.) doses of F1-V and then boosted with either adjuvanted s.c. doses (s.c. F1-V mice) or unadjuvanted oral doses (oral F1-V mice). Oral F1-V mice had higher prechallenge serum immunoglobulin G1 (IgG1) titers than s.c. F1-V mice. The corresponding serum levels of antigen-specific IgG2a and IgA were 2 and 3 orders of magnitude lower, respectively. After vaccination, mice were exposed to an inhaled dose of 1.02×10^6 CFU of aerosolized *Yersinia pestis* CO92 (50% lethal dose, 6.8×10^4 CFU). All control animals died within 3 days. F1-V given s.c. (with adjuvant) protected 33% of the immunized mice, while 88% of the oral F1-V mice survived aerosolized *Y. pestis* challenge. A comparison of splenic *Y. pestis* CFU counts showed that there was a 7- to 10-log reduction in the mean bacterial burden in survivors. Taken together, these data indicate that oral booster doses effectively elicit protective immune responses in vivo. In addition, this is the first report of a plant-derived oral vaccine that protected animals from live *Y. pestis* challenge, bringing the likelihood of lower-cost vaccines closer to reality.

The establishment of successful protocols for oral vaccination could radically alter the current landscape of infectious diseases. Oral delivery of plant-derived vaccine antigens could eliminate expensive fermentation and purification systems, cold storage and transportation steps, and delivery via sterile needles, significantly reducing costs. Plant-derived oral vaccines have other distinct advantages, including the ability to stimulate both systemic and mucosal immune responses, facilitating large-scale production and simplified storage (eliminating frozen stocks), improving safety due to the lack of human pathogens or microbial toxin contamination, protecting therapeutic proteins by bioencapsulation, and delivering these proteins to the gut-associated lymphoid tissue (7, 15, 80).

The engineering of chloroplasts for the production of vaccines and biopharmaceuticals has ushered in a new era in biotechnology (15, 17, 44). Chloroplast transgenes can express large amounts of foreign proteins (up to 46% of the total leaf protein [19]). Transgene silencing does not occur in chloroplasts at the levels of transcription and translation (19, 20). Chloroplasts translate heterologous operons, processed monocistrons, and unprocessed polycistrons (67), enabling the expression of multivalent vaccines. Most important, chloroplast transformation offers transgene containment via maternal inheritance; expression in leaves or vegetative organs eliminates transmission of transgenes in reproductive structures (14, 16). Additional containment methods, such as cytoplasmic male sterility, have also been developed using the chloroplast genome (71). Foreign proteins expressed in chloroplasts are protected in the digestive tract and are efficiently delivered to the circulatory system (52). Plastid transformation of edible crops, including soybean, carrot, and lettuce, has been accomplished recently (22, 45, 49). Moreover, human therapeutic proteins have been stably expressed in lettuce chloroplasts; oral delivery of proinsulin expressed in chloroplasts protected nonobese diabetic mice against the development of insulitis (70). These advancements have opened the door for developing vaccine antigens that can be orally administered.

Several vaccine antigens have already been expressed in chloroplasts, including the cholera toxin B subunit of *Vibrio cholerae* (18), *Bacillus anthracis* protective antigen (47, 87), LecA from *Entamoeba histolytica* (11), the 2L21 peptide from the canine parvovirus (59), and TetC of *Clostridium tetani* (83). These antigens were evaluated mostly by using conventional vaccination strategies, i.e., needle-based subcutaneous (s.c.) or intraperitoneal delivery. Therefore, further studies are required to evaluate and understand the mechanism of oral delivery of chloroplast-derived vaccine antigens in plant cells.

Yersinia pestis has caused three plague pandemics and killed approximately 200 million people (62). At least 2,000 cases of plague are reported annually by the World Health Organization (http://www.who.int/mediacentre/factsheets/fs267/en/index.html),

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including several recent outbreaks in India. The nonavailability of a human plague vaccine is a public health concern, given the potential use of *Y. pestis* as an agent for bioterrorism (42). Since the lungs and respiratory tract are vulnerable to a first exposure to *Y. pestis*, adequate protective immunity—achieved either by transudation of high levels of circulating immunoglobulin G (IgG), by induction of local immunity (IgA), or by a combination of both—must be available. Indeed, protection against both s.c. (95) and aerosolized (3, 93) *Y. pestis* challenges was found to be associated with F1-V-specific IgG1, a TH2-associated antibody. Systemic IgG is a known consequence of parenteral immunization, and many studies have demonstrated the efficacy of s.c. and intramuscular vaccines for providing protection against pathogen challenge (for a review, see reference 94). However, intranasal or even oral delivery of subunit vaccines may be more effective because of the ability of such vaccines to elicit protective immunity directly at the mucosal surface.

Human plague vaccines based on either a live, attenuated strain or a killed, whole-cell preparation (for a review, see reference 5) are no longer commercially available. Because of the severity of the infection and the potential of *Y. pestis* as a bioterrorist agent, we developed a subunit vaccine produced in transgenic tobacco chloroplasts. This vaccine offers the advantage of employing two defined antigens that are able to elicit high-level protection. Of the various *Y. pestis* antigens that have been tested preclinically, the fraction 1 (F1) outer capsular and low-calcium response V (LcrV or V) proteins appear to be the most promising vaccine candidates (9, 31, 64). The F1 protein has been reported to have antiphagocytic capability (21, 68, 89), while LcrV, a major component of the type III secretion system, is required for the production and translocation of *Yersinia* outer proteins, several of which have antihost activities in the eukaryotic host cell (63, 77, 79).

Given the high costs associated with needle-based vaccination, we investigated whether needle-free (i.e., oral) vaccine delivery could provide animals with similar levels of protection against pathogen challenge. We hypothesized that a heterologous prime-boost strategy for plague may provide improved protection compared with parenteral immunization. We investigated the efficacy of a plague vaccine protocol that incorporates both elements of successful vaccination against *Y. pestis*: s.c. delivery of enriched antigen preparations of F1-V prepared from transgenic, low-nicotine tobacco (enF1-V), followed by oral boosts of antigen expressed in transgenic plants. The efficacy of the vaccine was assessed by aerosol challenge with *Y. pestis*.

However, the intent of this study was not to compare oral boosters with s.c. boosters (by dosage or number of boosters). Experimentally, any such comparison is not possible because oral delivery involves antigens encapsulated in plant cells without any adjuvant, whereas in s.c. delivery antigens bound to an adjuvant are directly delivered to the circulatory system. It is not possible to deliver antigens via oral boosters in a quantitative manner because the release of antigens from plant cells depends on several factors, including the population of bacteria that can degrade the plant cell wall and presentation of an antigen to the gut-associated lymphoid tissue. It is not possible to control these factors in experimental animals in a quantitative manner. An equal number of boosters does not guarantee an equal quantity of antigen delivered. Therefore, this study simply demonstrated that oral boosting via plant cells is a novel mode of delivery of vaccine antigens and might provide a low-cost delivery option, especially for delivery of biodefense vaccines at times of crisis to a very large population or in developing countries where cold storage and transportation of vaccines are major challenges.

MATERIALS AND METHODS

Construction of pLDS-F1V and regeneration of transgenic lines. The F1-V fusion gene in a pET-24 vector (pPW731, obtained from the United States Army Medical Research Institute of Infectious Diseases [USAMRIID]) was isolated by cleavage with the restriction enzymes NdeI and NotI. The fragment was subcloned into a pCR vector containing the 5′ untranslated region (UTR) of the *psbA* gene and then inserted (using NotI and EcoRV) into the universal chloroplast vector pLD to create pLDS-F1V. The chloroplast expression vector pLDS-F1V was bombarded into Petit Havana and LAMD (low-nicotine variety) *Nicotiana tabacum* leaves as described elsewhere (48, 85, 86). Transgenic shoots were first tested by PCR to confirm transgene integration. Plants that were confirmed to contain the F1-V transgene were transferred to pots and were grown with a photoperiod consisting of 16 of h light and 8 h of darkness in a growth chamber at 26°C or in a greenhouse.

Southern blot analysis. Total plant DNA was extracted from tobacco plants using a DNeasy plant mini kit (Qiagen, Valencia, CA). Total plant DNA was digested with BamHI and hybridized with the flanking sequence probe, which was obtained from the pUC-Ct vector by digesting it with BamHI and BglII, which yielded a 0.81-kb fragment. The probe was prepared by random primed ^{32}P labeling (Ready-To-Go DNA labeling beads; Amersham Biosciences, Pittsburgh, PA). The probe was hybridized to the membrane using the Quick-hyb solution and protocol (Stratagene, La Jolla, CA). The radiolabeled blots were exposed to X-ray film and then developed.

Immunoblot analysis. The immunoblot analysis protocol has been described previously (48, 85, 86). Plant extraction buffer (PEB) (48, 85, 86) was made fresh on the day of the analysis. Extraction was performed using a ratio of 100 mg of leaf material to 200 μl of PEB. Transgenic protein was detected using polyclonal serum raised against F1 in rabbits (USAMRIID).

ELISA. Dilutions of plant crude extracts ranging from 1:5 to 1:5,000 were made in coating buffer (48, 85, 86). Recombinant F1-V (standard) was also diluted in coating buffer. An indirect ELISA was performed as described previously (48, 85, 86). The transgenic protein was detected using the anti-F1 polyclonal primary antibody.

Estimation of TSP. The total soluble protein (TSP) in plant crude extracts was determined by the Bio-Rad protein assay as previously described (48, 85, 86). Bovine serum albumin (Sigma Chemical, St. Louis, MO) was used as a standard at concentrations ranging from 0.05 to 0.5 mg/ml.

Lyophilization and enrichment of transgenic crude extracts. To concentrate the soluble protein in transgenic leaf material, 28.99 g of transgenic leaf material was extracted in 75 ml of PEB. Aliquots (10 ml) were transferred to 50-ml conical tubes and lyophilized to obtain a final volume of 1.5 ml. The concentrated extracts were then pooled, loaded onto Centricon 50-kDa molecular weight cutoff (MWCO) columns (Millipore, Billerica, MA), and centrifuged for 10 min at 5,000 × *g*. The flowthrough fraction was collected and run through the same column a second time. The retentate fractions were collected, pooled, and loaded onto 100-kDa MWCO columns, and the process was repeated. All flowthrough and retentate fractions were analyzed for the presence of F1-V by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot analysis, and ELISA.

Adsorption of enF1-V protein to adjuvant. F1-V enriched from transgenic tobacco crude extract (enF1-V) was mixed with Alhydrogel (AlH) (Sigma Chemical, St. Louis, MO) diluted 1:4 in phosphate-buffered saline (PBS) and incubated at 4°C with gentle rocking overnight. The samples were then centrifuged at 2,000 × *g* for 5 min at 4°C, and the protein-adsorbed pellet was resuspended in PBS to a final concentration of 250 μg/ml. The adsorption efficiency was calculated on the basis of the total amount of protein added to the adjuvant compared with the protein remaining in the supernatant after adsorption.

Immunization. Female Hsd:ND4 Swiss Webster mice weighing 18 to 20 g each were purchased from Harlan Sprague Dawley (Indianapolis, IN). Mice were divided into the following five treatment groups: group 1, mice given s.c. enF1-V prime and s.c. enF1-V boost doses (s.c. F1-V group) (10 animals); group 2, mice given s.c. enF1-V prime and oral F1-V boost doses (oral F1-V group) (10 animals); group 3, mice given s.c. enF1-V prime and oral wild-type boost doses (oral WT group) (10 animals); group 4, mice given s.c. AlH prime and s.c. AlH

boost doses (s.c. AlH group) (5 animals); and group 5, untreated mice (5 animals).

s.c. injections were delivered as follows. Doses of chloroplast-derived enF1-V adsorbed to AlH were diluted in 200 μl of PBS and injected into the scruff of the neck using a tuberculin syringe equipped with a 27-gauge needle. A single 25-μg dose of enF1-V was delivered on day 0 as the vaccine prime dose to animals in groups 1 to 3; boosts of 10 μg of enF1-V per dose were given to animals in group 1. Group 4 animals received equivalent amounts of AlH in the absence of F1-V. Mice in groups 1 and 4 received four s.c. boosts, on days 14, 28, 126, and 164.

The doses used for oral delivery were prepared as follows. Leaf material from transgenic or wild-type plants was ground in liquid nitrogen in cold, autoclaved mortars and pestles. The pulverized leaf material was stored at −80°C until the day of immunization. Oral doses (500 mg each) of either transgenic (group 2) or nontransgenic (group 3) leaf material were resuspended in sterile PBS (250 μl) and homogenized for 5 min with an OMNI International GLH-2596 probe to disperse the plant cells. The plant cell suspension (without clumps) was stored on ice until oral gavage. The oral doses were delivered by using a tuberculin syringe equipped with a 20-gauge bulb-tipped gastric gavage needle. Mice in groups 2 and 3 received eight oral boosts, on days 8, 15, 22, 29, 119, 126, 164, and 171. Mice were shipped to USAMRIID on day 182.

Determination of antibody titers. Blood samples were obtained on day 7 before vaccination and days 21, 43, and 140 after vaccination. Blood was collected from the retroorbital plexus of anesthetized mice (4% isoflurane) in Microtainer serum separation tubes (Becton-Dickinson, Franklin Lakes, NJ). The samples were allowed to clot for a minimum of 30 min at room temperature and then centrifuged at 13,000 × *g* for 2 min. The serum was transferred to fresh tubes and either placed on ice or stored at −80°C.

Serum levels of F1-, V-, and F1-V-specific IgG1 and F1-V-specific IgG2a and IgA were determined by ELISA. Purified recombinant F1, V, or F1-V (100 ng), diluted in coating buffer, was incubated overnight at 4°C. Fivefold dilutions of serum (beginning with 1:100 in PBS) were then aliquoted and incubated overnight at 4°C. The secondary antibody was either anti-mouse IgG1 or IgG2a or IgA. To compare the levels of antibodies of different isotypes, dilutions of purified IgG1, IgG2a, and IgA were incubated overnight at 4°C, followed by addition of secondary antibody (anti-IgG1, anti-IgG2a, and anti-IgA, respectively).

Bacterial challenge. *Y. pestis* CO92 was prepared and used in accordance with a previously reported procedure (2). Briefly, bacteria were harvested from tryptose blood agar base (Difco Laboratories, Detroit, MI) slants and inoculated into 5 ml of heart infusion broth (HIB) (Difco), and the concentration was adjusted to an optical density at 620 nm of 1.0 (approximately 10^9 CFU/ml). For aerosol challenges, 2 ml of the HIB bacterial suspension was used to inoculate flasks containing 100 ml HIB supplemented with 0.2% xylose. The broth cultures were grown for 24 h in a 28°C shaker at 100 to 150 rpm. The concentration of pelleted cells was adjusted to an optical density at 620 nm of 10.0 (approximately 10^{10} CFU/ml), and the preparations were diluted to produce the aerosolized doses reported below. Antifoam agent A (Sigma) was added to a final concentration of 0.2% (vol/vol) to the bacterial suspension just before the aerosol challenges. The aerosol challenges were conducted by whole-body exposure of mice to a small-particle aerosol (median diameter, 1.2 μm). Up to 40 unanesthetized mice were challenged simultaneously inside a class III biological safety cabinet. Mice from various groups were divided into different cages to minimize exposure differences. The inhaled doses for each exposure were estimated using Guyton's formula (33). Mice were observed twice daily for 21 days after exposure for signs of morbidity or mortality. Any mouse found to be recumbent was humanely euthanized.

Calculation of bacterial burden. Homogenates (10%) of whole spleen tissue were plated in duplicate at the indicated dilutions on sheep blood agar plates. The plates were incubated at 28°C for 48 h, and colonies were counted. The total bacterial burden per gram of spleen was calculated by multiplying the plate count by the appropriate dilution factor.

Statistical analysis. Pearson analysis was conducted to calculate correlation coefficients comparing survival postchallenge and individual mouse day 140 postprime immunization F1-V-specific antibody titers. Fisher exact tests with step-down Bonferroni adjustments were used to compute statistical differences between observed survival rates. Survival curves were processed using Kaplan-Meier survival analysis with log rank tests and step-down Bonferroni adjustments. Statistical significance was considered a P value of <0.05, as indicated below.

Care and treatment of animals. This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The animals received food and water ad libitum for the duration of the study.

RESULTS

Assessment of transgenic plants. The F1-V fusion gene was cloned into the universal chloroplast vector pLD-CtV to produce the final vector, pLDS-F1V (Fig. 1A), which was used for particle bombardment. In this construct, the *trnI* and *trnA* genes were used as flanking sequences for homologous recombination with the native chloroplast genome. The *aadA* gene conferring spectinomycin resistance was used for selection. True chloroplast transformants were distinguished from nuclear transformants and mutants by PCR (data not shown). PCR was also employed to ensure integration of the F1-V fusion gene (data not shown). Confirmed Petit Havana and LAMD (12) transgenic shoots were transferred to rooting medium and then to either growth chambers or the greenhouse. In order to evaluate homoplasmy, total DNA extracted from untransformed and transgenic lines was probed with chloroplast flanking sequences (Fig. 1A). Homoplasmic lines of both cultivars contained both 9.5- and 1.5-kb fragments without the 8-kb fragment observed in untransformed lines (Fig. 1B).

The light-regulated 5′ UTR and promoter of the *psbA* gene were used to enhance transcription and translation; the 3′ UTR conferred transcript stability. The prokaryotic chloroplast favors AT-rich sequences, which reflects the tRNA abundance. Therefore, the F1-V fusion gene, containing 65% AT, was expected to be expressed well in chloroplasts. Mature leaves always had the highest level of expression of F1-V, and the highest content was on day 3 with continuous illumination (an average of 14.8% of the TSP) (Fig. 2). The *psbA* 5′ UTR accounted for both the high expression of F1-V and the change in expression during continuous illumination.

Enrichment of F1-V. Pooled chloroplast transgenic lines containing a mixture of young, mature, and old leaves were further analyzed for protein expression. Crude extracts of 223 mg of transgenic leaf material contained about 11 mg/ml of TSP or 404 μg/ml of F1-V, equivalent to 3.68% of the TSP or 1.01 mg F1-V per g of freeze-dried leaf material. The lyophilization procedure did not degrade the F1-V protein; no cleaved product was detected in lyophilized crude extracts by immunoblot analysis (Fig. 3A). The lyophilized extract was then enriched for F1-V using MWCO columns. Immunoblot analysis of the various fractions indicated that F1-V was present in the >100-kDa fraction but not in the 50- to 100-kDa fraction (Fig. 3B). This may have been due to protein aggregation, which is commonly observed in transgenic leaf extracts due to high concentrations. Alternatively, it has been reported that during purification F1-V antigens exist principally as dimers and tetramers when they are expressed in *E. coli* (66). Dilutions of enF1-V were analyzed by SDS-PAGE, and there was a prominent band at ~53 kDa (Fig. 3C), corresponding to the size of the F1-V fusion protein. A comparison of the F1-V content of leaf crude extracts with chloroplast-derived enF1-V demonstrated that the enrichment procedure resulted in a nearly 80% improvement in the concentration of transgenic protein in the sample (3.68% versus 18.23% of the TSP).

Animal vaccinations. Mice were distributed across five treatment groups, four of which received primary s.c. vaccinations.

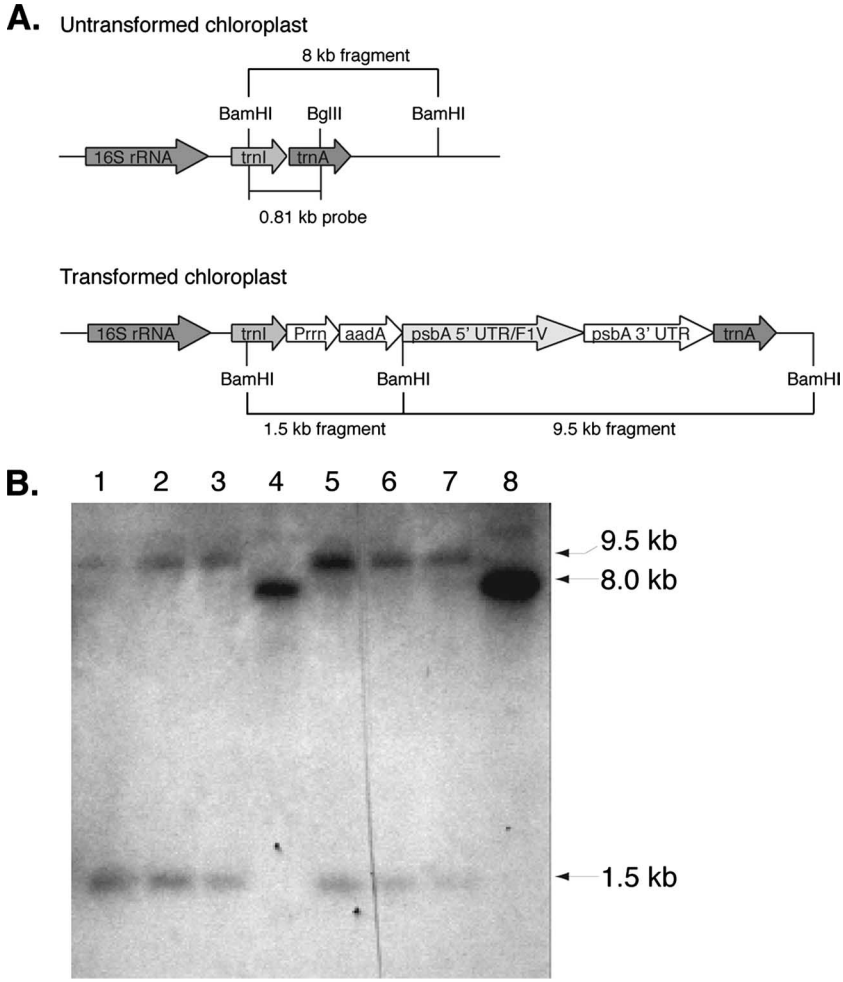

FIG. 1. Evaluation of transgene integration and homoplasmy in the T_0 (first) generation of both cultivars. DNA extracted from transformed and untransformed plants were probed with the flanking sequence probe (A). Southern blot analysis of transgenic lines in lanes 1 to 3 (Petit Havana) and lanes 5 to 7 (LAMD) produced restriction fragments that were 9.5 and 1.5 kb long (B). Wild-type Petit Havana (lane 4) and LAMD (lane 8) plants produced only the 8-kb fragment.

s.c. doses contained enF1-V adsorbed to AlH as an adjuvant, which has been shown to increase the availability and stability of an antigen(s) (32). Each of the treated animals received an s.c. priming dose, which served to induce an initial immune response in groups 1 to 3 to F1-V. Groups of mice received either four s.c. boosts or eight oral boosts. Oral doses (oral F1-V group) were delivered twice as frequently as s.c. doses because of the reduced efficiency of antigen processing by the gut mucosa (72). In addition, as s.c. boosts were delivered with an adjuvant, doubling the number of oral doses was considered adequate compensation for the absence of mucosal adjuvant.

We determined the serum titers of antigen-specific IgG1, IgG2a, and IgA antibodies at various time points. Although mice were immunized with the F1-V fusion protein, we assessed the IgG1 levels based on specificity to three discrete antigens, F1, V, and F1-V. No animal had detectable levels of F1-, V-, or F1-V-specific IgG1 in its preimmune serum (Fig. 4A, 4B, and 4C, respectively), indicating that there was no prior exposure to *Y. pestis*. There was no statistically significant difference between the F1- and V-specific IgG1 titers at any of the time points tested ($P > 0.05$ in all cases).

Following the primary immunization mice received a series of either four s.c. or eight oral boosts. Throughout the schedule, there was an increase in the IgG titers in vaccinated mice, which peaked at day 140 (Fig. 4). The only exception was F1-specific IgG1, whose titers were similar at all time points tested (Fig. 4A). An analysis of the binding affinities of the secondary antibodies indicated that the anti-IgG1 antibody bound purified IgG1 with approximately 40% greater affinity than it bound the IgG2a antibody pair (data not shown). Serum antibody titers were therefore adjusted to reflect the different binding affinities.

Mice in the s.c. F1-V and oral F1-V groups had significantly higher (~2 logs) serum F1-V-specific IgG1 titers than IgG2a titers (compare Fig. 4C and 4D). The development of high titers of circulating IgG1 antibody was associated with protection ($r = 0.71$), while the development of circulating IgG2a antibody was not ($r = 0.15$). Of the three mice in the s.c. F1-V

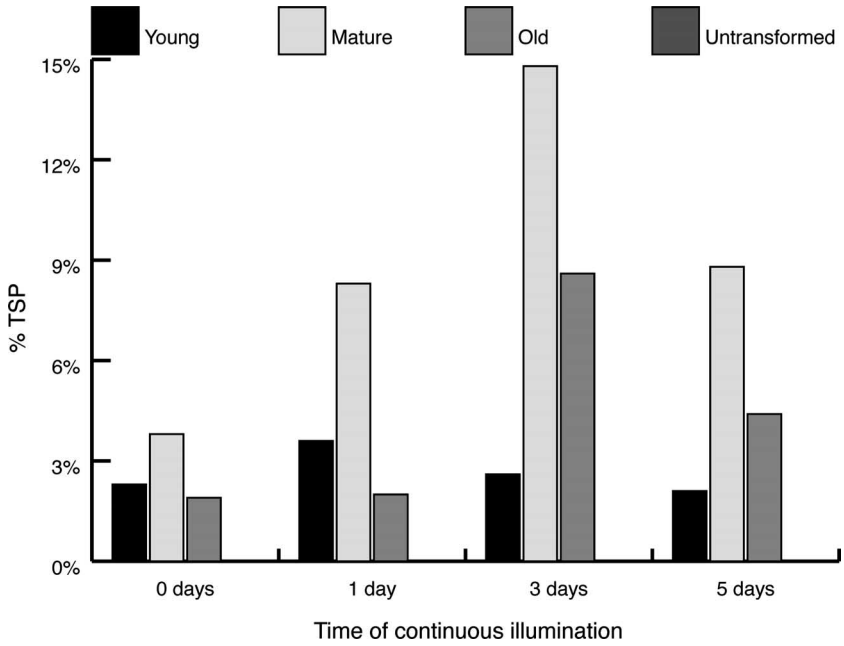

FIG. 2. Quantitation of chloroplast-synthesized F1-V by ELISA. The quantity of F1-V is expressed as a percentage of the TSP. For continuous illumination, leaf material was sampled on days 0, 1, 3, and 5 for young, mature, and old leaves.

group with the highest day 140 IgG1 titers, two survived (Fig. 4C and Table 1). Fecal IgA levels were also assessed and were below the limit of detection for most animals (data not shown). However, we did observe serum levels of F1-V-specific IgA (Fig. 4E). The IgA titers did not vary significantly ($P > 0.05$) between groups and were weakly correlated with protection ($r = 0.26$).

Aerosol challenge. The 50% lethal dose (LD_{50}) for a Swiss Webster mouse for whole-body exposure to aerosolized *Y. pestis* CO92 is 6.8×10^4 inhaled CFU. The calculated inhaled dose for each mouse was 1.02×10^6 CFU or 15 LD_{50}. Doses in this range have been used previously for successful aerosol *Y. pestis* challenge (3, 36, 75).

Mice were challenged on day 189. Following aerosol challenge, mice were observed twice daily for *Y. pestis*-dependent morbidity and mortality. The challenge dose was sufficient to induce death in untreated control animals, and the mean time to death (MTD) was 3 days (Fig. 5), which is consistent with our previous observations for untreated mice (35). Gross pathological examination of mice that succumbed to the challenge revealed significant lung damage consistent with primary plague pneumonia (data not shown). Lungs of mice that survived until the end of the observation period appeared to be grossly normal (data not shown).

All control animals died as a result of pathogenic *Y. pestis* challenge by day 8 postchallenge. At that time, three of nine mice (33%) that received enF1-V delivered as s.c. boosts with adjuvant survived (Fig. 5). The protection was statistically significant compared to mice that received adjuvant only ($P = 0.0438$) or untreated controls ($P = 0.0411$). Seven of eight mice (88%) in the oral F1-V group survived until the end of the challenge experiment (day 22), even without adjuvant. The single mouse that succumbed during the observation period died on day 14 (while the MTD for control animals was 3 days), and this animal had a bacterial count of 1.60×10^7 CFU/g, which was several logs less than the count for control animals (mean bacterial count, 2.17×10^{10} CFU/g). Both survival and MTD were statistically significant when the mice were compared to all three control groups ($P < 0.0001$). There was also a statistically significant difference between the oral F1-V and s.c. F1-V groups in terms of survival rate and MTD ($P < 0.0001$). Mice that survived the observation period were found to be free of infection by direct plating of spleen tissue (Table 1). The plant-derived vaccine appeared to reduce the bacterial burden in vaccinates that did succumb to infection. A comparison of the *Y. pestis* CFU counts for the spleens of control animals and vaccinates showed that there was an approximately 2-log reduction in the mean bacterial burden of the vaccinates (Table 1).

DISCUSSION

This study demonstrated that oral booster doses of *Y. pestis*-derived antigens were at least as effective at eliciting protective antigen-specific antibody responses as needle-based s.c. doses of the same antigens. In addition, we found for the first time that a plant-based vaccine against the etiologic agent of plague successfully protected mice from lethal *Y. pestis* challenge. The levels of F1-V in chloroplasts—up to 14.8% of the TSP—enabled the delivery of sufficient amounts of vaccine antigens in intact plant cells.

We observed that oral boosts of transgenic plant material containing the plague fusion antigen F1-V without adjuvant performed as well as s.c. boosts containing chloroplast-derived enF1-V with adjuvant at eliciting a predominant IgG1 titer in the serum of vaccinates. This type of response is indicative of an ongoing TH2 response (78) and, in s.c. vaccinated animals, is typical of AlH-adjuvanted vaccines (32, 53). The TH2 re-

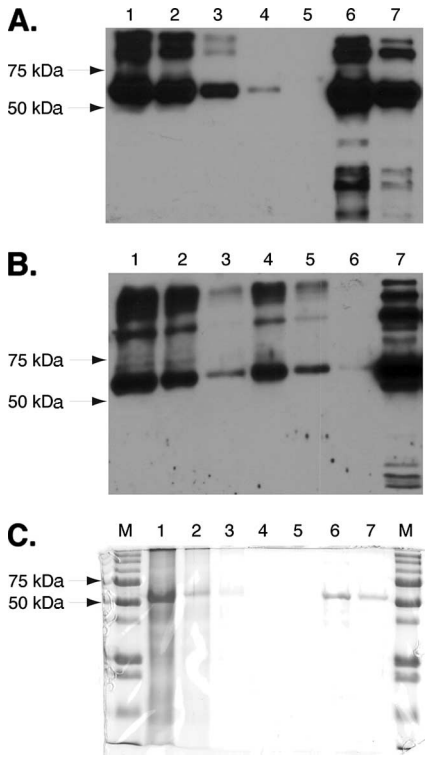

FIG. 3. Enrichment of the chloroplast-derived F1-V antigen. Transgenic plant crude extracts were lyophilized and then sequentially centrifuged through 50- and 100-kDa MWCO columns. (A) Immunoblot analysis of lyophilized crude extracts. Lanes 1 to 5, dilutions of lyophilized crude extract (1:1, 1:10, 1:100, 1:1,000, and 1:5,000); lanes 6 and 7, 750 and 250 ng, respectively, of recombinant F1-V purified from *E. coli*. (B) Immunoblot analysis of enriched plant extracts analyzed by SDS-PAGE, followed by immunoblotting. Lanes 1 to 3, dilutions of >100-kDa column retentate (1:5, 1:10, and 1:50); lanes 4 to 6, dilutions of >100-kDa column wash (1:5, 1:10, and 1:50); lane 7, 750 ng of recombinant F1-V purified from *E. coli*. (C) Chloroplast-derived enF1-V samples were analyzed by SDS-PAGE, followed by staining with Coomassie blue. Lanes 1 to 5, dilutions of lyophilized crude extract (1:5, 1:10, 1:100, 1:500, and 1:50,000); lanes 6 and 7, 750 and 250 ng, respectively, of recombinant F1-V purified from *E. coli*; lanes M, molecular weight markers.

sponse, specifically mediated by serum levels of F1-V-specific IgG1, has been shown to protect against both s.c. (95) and aerosolized (3, 93) *Y. pestis* challenge. Our results corroborate these findings. Orally boosted mice had similar or, in some cases, higher levels of antigen-specific IgG1. Further, these responses were consistent across the various antigens tested, F1, V, and F1-V. The anti-F1 and anti-V IgG1 responses were not significantly different from one another ($P > 0.05$), indicating that the individual components of the F1-V fusion protein had relatively equal immunogenicities.

The IgG1 levels were generally 2 to 3 logs higher than the corresponding IgG2a levels. Not surprisingly, animals with the highest IgG1 titers were more likely to survive challenge with live *Y. pestis* ($r = 0.71$) (Table 1). Overall, oral F1-V boosters yielded somewhat higher IgG1 titers, more survivors, and a longer MTD than s.c. F1-V boosters. Oral WT group control-boosted mice had the lowest IgG1 titers of the three vaccinated mice, the fewest survivors, and the shortest MTD. Collectively, these results may suggest that the route of immunization or the adjuvant plays a critical role in driving the formation of a protective response in which both IgG1 and IgG2a are present. It is important to note, however, that the oral boosts in our study did not contain adjuvant, nor was the acidic pH of the gut neutralized, which has been done in other oral vaccination schemes (50, 96).

Because of the severe pathogenicity of *Y. pestis*, treatment of plague is a high priority. The use of antimicrobial agents began in 1938 (65) and has led to a dramatic drop in human mortality. Today, the worldwide fatality rate attributable to plague has fallen to less than 8% (26). Natural isolates of *Y. pestis* are uniformly susceptible to all antimicrobial agents active against gram-negative bacteria (8, 26). However, a "natural" strain resistant to multiple antibiotics was isolated in 1995 in the Ambalavao district of Madagascar (27). The possibility of the occurrence of such multidrug-resistant strains in the natural environment, the ease of generating such strains under laboratory conditions (39, 41), and the potential use of such strains for bioterrorist attack, together with the rapidity and high lethality of the disease (for a review, see reference 5), indicate that it is necessary to search for alternatives to antibiotics.

Immunization is now one of the major approaches being pursued to deal with potential *Y. pestis* infection. Use of the serum of vaccinated rabbits to cure animals infected with *Y. pestis* was first attempted more than 100 years ago (97). Since then, several antigens have been shown to be able to produce protective immunity. Among these antigens are the F1 capsular (58, 76) and LcrV (or V) antigens (51, 61, 84), both of which also contain immunodominant epitopes (38, 73, 74, 98). Passive administration of antibodies against target antigens protects macrophages from *Y. pestis*-induced cell death, promotes phagocytosis (13, 64, 88), and protects animals against both bubonic plague and pneumonic plague (4, 25, 37, 61, 69, 84, 91). However, therapy based on a single antibody against a single antigen or epitope will be ineffective in the case of infection with a virulent strain lacking the antigen or expressing a different serological variant of the antigen (6, 25, 69).

Human plague vaccines based on either a live, attenuated strain or a killed, whole-cell preparation (for a review, see reference 5) are no longer commercially available. Given the severity of the infection and the potential of the organism as a bioterrorist agent, we describe a subunit vaccine produced in transgenic tobacco chloroplasts. This vaccine offers the advantage of employing two defined antigens, F1 and V, that are able to elicit high-level protection. Subunit vaccines have been shown to be less reactogenic than the former whole-cell vaccines (6, 90, 91).

There have been several reports of s.c., intranasal, and oral vaccination strategies protecting animals from *Y. pestis* challenge (for a review, see reference 82). Oral plague immunization studies of attenuated *Salmonella* vaccines have shown that these vaccines are effective (28, 60, 81). But there are clinical concerns for vaccination of special-needs populations, such as the young, the old, pregnant women, and immunocompromised individuals. Another practical concern is plasmid maintenance and stability. We believe that the use of orally delivered plant-based vaccines may obviate these concerns and that such vaccines may be less reactogenic in the general population.

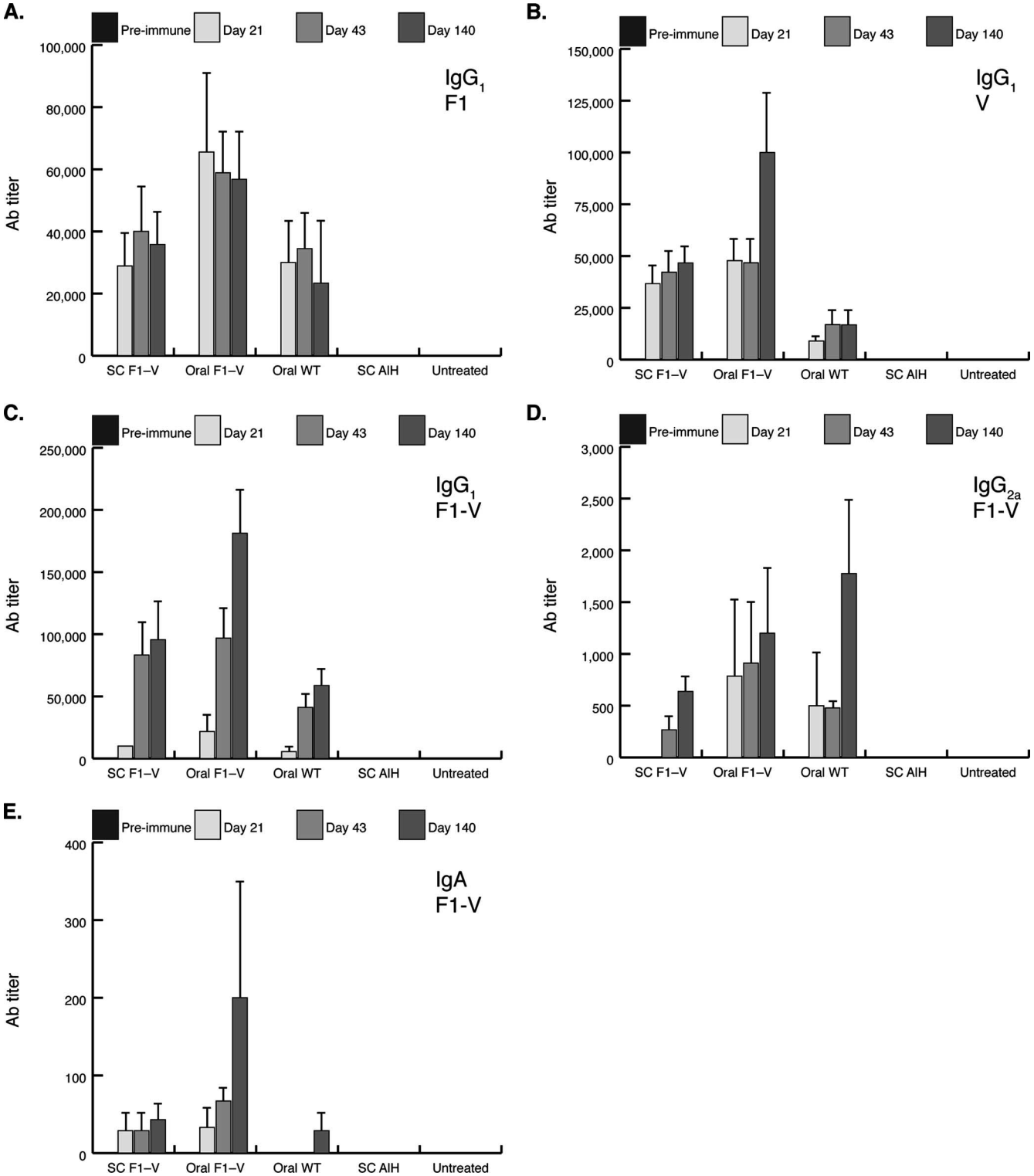

FIG. 4. Quantitation of serum antibody titers after immunization. Mice were immunized as described in the text. On days 21, 43, and 140, blood was collected and the serum was analyzed for the presence of (A) F1-specific IgG1, (B) V-specific IgG1, (C) F1-V-specific IgG1, (D) F1-V-specific IgG2a, and (E) F1-V-specific IgA antibodies. IgG titers were calculated by determining the reciprocal of the highest dilution that resulted in a difference between each treatment group and untreated group of 0.25 optical density unit; IgA titers were calculated based on a difference between each treatment group and untreated group of 0.1 optical density unit. SC F1-V, s.c. enF1-V prime dose and s.c. enF1-V boosts; Oral F1-V, s.c. enF1-V prime dose and oral F1-V boosts; Oral WT, s.c. enF1-V prime dose and oral wild-type boosts; SC AlH, s.c. AlH prime dose and s.c. AlH boosts. Ab, antibody.

TABLE 1. Antibody titers and bacterial (*Y. pestis*) counts for immunized mice

Group	Mouse	Day of death after challenge	Antibody titer[a]			*Y. pestis* concn (CFU/g)[b]	
			IgG1	IgG2a	IgA	Spleen	Group mean
1 (s.c. F1-V)	1.1	—[c]	100,000	1,000	100	0	0
	3.1	—	50,000	500	0	0	
	7.1	—	250,000	500	0	0	
2 (oral F1-V)	1.2	—	50,000	500	100	0	0
	2.2	—	250,000	5,000	1,000	0	
	2.4	—	250,000	2,500	0	0	
	3.2	—	250,000	500	100	0	
	5.2	—	250,000	1,000	0	0	
	6.2	—	250,000	1,000	0	0	
	7.2	—	250,000	1,000	1,000	0	
4 (s.c. AlH)	1.5	3	0	0	0	6.02×10^{10}	2.17×10^{10}
	2.5	3	0	0	0	1.27×10^{10}	
	3.5	3	0	0	0	1.30×10^{10}	
	7.4	5	0	0	0	9.00×10^{8}	
5 (untreated)	5.5	3	0	0	0	1.94×10^{10}	1.39×10^{10}
	6.5	3	0	0	0	1.05×10^{10}	
	7.5	3	0	0	0	6.30×10^{9}	
	8.5	3	0	0	0	1.94×10^{10}	
1 (s.c. F1-V)	1.4	6	50,000	0	100	1.60×10^{9}	8.00×10^{8}
	8.1	6	50,000	500	0	5.60×10^{5}	
2 (oral F1-V)	4.2	13	50,000	500	0	1.60×10^{7}	1.60×10^{7}
3 (oral WT)	3.3	3	10,000	1,000	0	3.70×10^{10}	1.85×10^{10}
	4.4	8	50,000	250	0	5.90×10^{5}	

[a] The antibody titer was assessed on day 140 after prime immunization.
[b] At the time of death, spleens were excised from representative animals of each group to determine the bacterial burden.
[c] —, animals euthanized on day 22 postchallenge had no symptoms of infection.

One might expect that protection of the mucosal surface would be critical against aerosolized *Y. pestis*. Mucosal protection is normally mediated by local production of secretory IgA (56). Although IgA is the predominant humoral defense mechanism at mucosal surfaces, local IgM and IgG and, in the lower respiratory tract, serum IgG can also contribute significantly to immune defense (40). Indeed, serum IgG1 has been shown to be the predominant isotype produced in response to recombinant plague vaccines and to be associated with protection against aerosolized *Y. pestis* (3, 91, 92). The high IgG1 titers elicited in this study confirmed these findings and indicate that there was appropriate immune modulation by our plant-based vaccine.

The contribution of IgA to the protective response to plague is less clear. Previous oral plague vaccine strategies have been shown to induce low levels of serum IgA, although the expression was not examined for an association with parenteral protection (60). In our study, we observed low but measurable levels of F1-V-specific IgA in the serum; however, this response correlated weakly with protection ($r = 0.26$). More recently, an attenuated *Salmonella* oral vaccine expressing both F1 and V antigens was shown to induce fecal IgA and serum IgG1/IgG2/IgG3 responses, as well as protection against both bubonic plague and pneumonic plague (96). Again, no direct comparison of IgA with survival was made. Another recent study in which F1-V was mixed with the mucosal adjuvant Protollin and administered intranasally did show that there was a statistical association between lung IgA levels and protection (43). This finding is complicated by the fact that there were also statistical associations between lung and serum IgG levels and protection. Collectively, these data suggest that an appropriate mucosal adjuvant may be required to induce secretory IgA. The ability to passively protect mice with IgA alone may need to be shown to firmly establish the contribution of IgA to overall protection from plague.

Other studies have suggested the importance of the route of immunization to the overall levels and types of antibody to F1 and V, as well as protection against subsequent *Y. pestis* challenge (23, 24). It is becoming increasingly clear that mixing of vaccine routes can have a positive effect on both antibody production and protection. It has been previously reported that in F1-V vaccinations, heterologous prime-boost regimens are at least as effective as homologous boost regimens for inducing serum anti-F1-V IgG1 responses (29, 30). In this study, all vaccinated mice received an initial s.c. injection, which effectively induced a potent systemic humoral response (Fig. 4). Oral delivery resulted in the highest levels of serum IgG1 and IgG2a of all groups but negligible titers of F1-V-specific IgA in the feces of animals. At first, this finding appears to be paradoxical. However, the route chosen for induction of peripheral immunity may delay or prevent the induction of mucosal immunity (10, 54, 55). Indeed, s.c. immunizations followed by oral boosts have been shown to suppress local intestinal antigen-specific IgA responses (34, 46). The latter

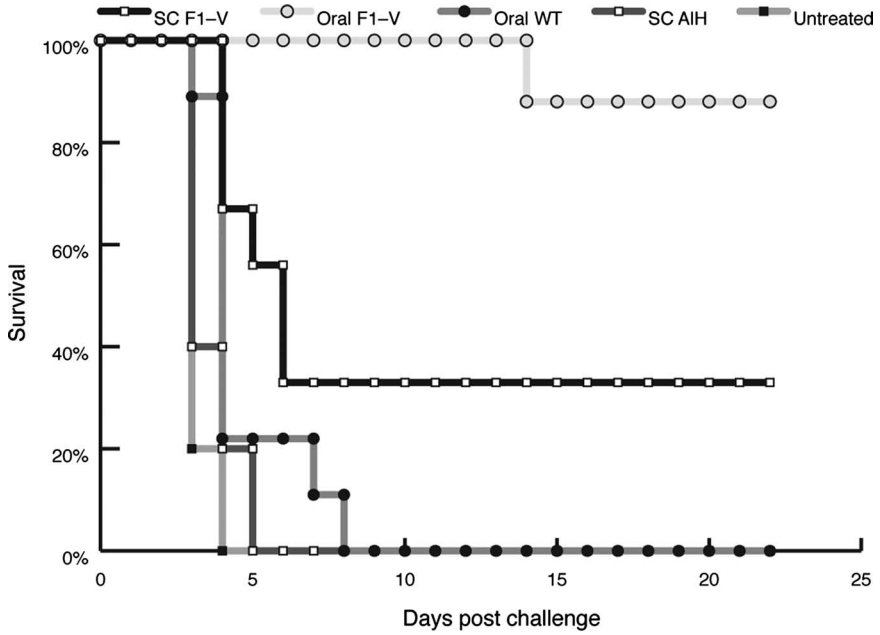

FIG. 5. Mice receiving oral boosts of chloroplast-derived F1-V survived longer than mice receiving s.c. boosts. Animals were challenged with 15 LD_{50} of *Y. pestis* CO92 (whole-body LD_{50}, 6.8×10^4 CFU), and their survival was monitored. Differences in survival between untreated animals and immunized animals were statistically significant ($P < 0.05$, as determined by the log rank test). Differences between animals boosted s.c. and animals boosted orally were also significant ($P < 0.05$). SC F1-V, s.c. enF1-V prime dose and s.c. enF1-V boosts; Oral F1-V, s.c. enF1-V prime dose and oral F1-V boosts; Oral WT, s.c. enF1-V prime dose and oral wild-type boosts; SC AlH, s.c. AlH prime dose and s.c. AlH boosts.

observation appears to support our finding that there was a limited IgA response following oral boosts of F1-V.

This report of a plant-based plague vaccine demonstrates for the first time that high-level protection of mice from the lethal pathogenic effects of aerosol challenge can be elicited by oral delivery. Virus-based plant expression systems have been used successfully to confer protection against *Y. pestis* challenge in guinea pigs and macaques upon s.c. immunization with purified F1-V antigens (57, 75). There is one previous report of an s.c. prime-oral boost strategy, involving *Agrobacterium*-transformed tomato nuclei, but no pathogen challenge was performed (1). The prime-boost strategy that we employed was designed to maximize antigen delivery to the immune system while minimizing processing time and costs. The efficacy of this strategy also indicates that oral immunization boosts are at least as effective as s.c. boosts for eliciting systemic humoral responses. In addition, if oral boosts are found to consistently perform at least as well as s.c. boosts, then the use of needle-based vaccines and the substantial costs associated with their use may need to be reexamined. Chloroplast technology is therefore an ideal system for oral delivery of vaccines.

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