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Protection against pneumonic plague following oral immunization with a non-replicating vaccine

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Abstract

Yersinia pestis is a dangerous bacterial pathogen that when inhaled can rapidly induce fatal pneumonic plague. Thus, there is a need for stable, safe, and easily administered mucosal vaccines capable of eliciting effective protection against pulmonary *Y. pestis* infections. Cationic liposome–nucleic acid complexes (CLDC) have been shown previously to be effective vaccine adjuvants for parenteral immunization, but have not been previously evaluated for use in oral immunization. Therefore, we investigated the ability of an orally administered CLDC adjuvanted vaccine to elicit protective immunity against lethal pneumonic plague. C57Bl/6 mice were vaccinated orally or subcutaneously using 10 µg *Y. pestis* F1 antigen combined with CLDC and immune responses and protection from challenge was assessed. We found that oral immunization elicited high titers of anti-F1 antibodies, equivalent to those generated by parenteral immunization. Importantly, orally immunized mice were protected from lethal pulmonary challenge with virulent *Y. pestis* for up to 18 weeks following vaccination. Vaccine-induced protection following oral immunization was found to be dependent primarily on CD4+ T cells, with a partial contribution from CD8+ T cells. Thus, CLDC adjuvanted vaccines represent a new type of orally administered, non-replicating vaccine capable of generating effective protection against pulmonary infection with virulent *Y. pestis*.

Keywords

Yersinia pestis; Antibody; T cells; Infection; Mucosal

1. Introduction

Yersinia pestis is a Gram-negative bacterium that causes severe infections in humans, including pulmonary infections that can result following inhalation of the organism [1–3]. A number of experimental vaccines have been developed for *Y. pestis* infection and most are based on immunization with *Y. pestis* F1 and V antigens, administered either alone or in

combination [4–8]. The F1 antigen is a glycoprotein that is a major component of the *Y. pestis* polysaccharide capsule and is one of the primary antigens used in vaccines against *Y. pestis* [8–11]. The V antigen comprises one subunit of the *Y. pestis* type III secretion system and is a second antigen commonly used for *Y. pestis* vaccines [12–15]. Parenteral immunization with F1 and V antigens can elicit effective protection against parenteral challenge with *Y. pestis* and in some cases may also generate protection against lethal inhalational challenge with *Y. pestis* [11,16–21].

Mucosally delivered vaccines are generally thought to provide more rapidly effective protection against pulmonary challenge with pathogens such as *Y. pestis* [8,22,23]. Thus, several different *Y. pestis* vaccines delivered to mucosal sites have shown promise in protection studies [20,21,24,25]. However, the only orally administered vaccines that have been shown to date to elicit protective immunity against inhaled *Y. pestis* have utilized live, replicating vaccine vectors. For example, oral immunization with a *Salmonella* vector engineered to over-express either F1 antigen alone or an F1/V antigen construct has been shown in several studies to elicit protective immunity against lethal *Y. pestis* challenge [11,26–28]. Protection against *Y. pestis* challenge has also been achieved with an orally administered, attenuated *Yersinia pseudotuberculosis* vaccine [29,30]. However, live-vectored vaccines have several drawbacks, including the need to assure full vector attenuation, the risk of disseminated vector replication in immunosuppressed individuals, and the need to maintain careful storage conditions to ensure vector viability [39]. Thus, non-replicating mucosal vaccines that were stable during storage and safe to administer would have several potential advantages over vectored vaccines.

The use of non-replicating, orally administered vaccines to elicit effective immunity against inhaled *Y. pestis* infection has not been reported previously. In previous studies we have shown that CLDC could be used as effective vaccine adjuvants to elicit strong CD8⁺ and CD4⁺ T cell responses following parenteral immunization [31,32]. In addition, preliminary data in our laboratory suggested that CLDC could also serve as effective adjuvants for orally delivered vaccines (Bosio and Dow, unpublished results). Therefore, in the present study we investigated whether CLDC could be utilized as adjuvants in orally delivered vaccines directed against *Y. pestis*. Here, we demonstrate that oral immunization with the *Y. pestis* glycoprotein F1 combined with CLDC adjuvant (F1/CLDC) generated effective and long-lasting protective immunity against primary pulmonary infection with virulent *Y. pestis*. Furthermore, we provide evidence that vaccination with F1/CLDC was associated with induction of strong humoral immunity and was found to be dependent on both CD4⁺ and CD8⁺ T cells. These studies suggested therefore that CLDC-based adjuvants may be used effectively for oral immunization.

2. Materials and methods

2.1. Mice

Specific pathogen-free wild type, CD4^{-/-}, and CD8^{-/-} mice on the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in sterile microisolate cages in the Laboratory Animal Resources facility at Colorado State University and were provided sterile water and food *ad libitum*. Challenge studies with *Y. pestis* were conducted in an approved BSL-3 facility at Colorado State University in accordance with Select Agent regulations and all research involving animals was approved by the Animal Care and Use Committee at Colorado State University.

2.2. Bacteria

Yersinia pestis strain Madagascar (MG05), which expressed both the F1 and V antigens of *Y. pestis*, was kindly provided by Dr. Martin Schrieffer (CDC, Fort Collins, CO). MG05 was grown in liquid brain-heart infusion (BHI) medium supplemented with 2.5 mM CaCl₂ at 26 °C for 24 h with shaking, then transferred to 37 °C with shaking for an additional 24 h. Bacteria were used immediately after cultivation and diluted in PBS prior to challenge to achieve a concentration of 1.5×10^6 CFU/ml.

2.3. Preparation of CLDC and F1 vaccine

Sterile complexes of cationic liposomes were prepared using equimolar amounts of DOTIM (octadecanoyloxy{ethyl-2-heptadecenyl-3-hydroxyethyl imidazolium chloride) and cholesterol, which were prepared as described previously, except that the liposomes were extruded through a final filter diameter of 200 nm rather than 100 nm [33]. The final plasmid DNA concentration in the complexes was 100 µg DNA per ml. Plasmid DNA was isolated from *E. coli* DH5a using the Qiagen Endo-free Giga kit according to the manufacturers instructions (QIAGEN, Valencia, CA). CLDC were formulated just prior to preparation of the vaccine by gently mixing cationic liposomes with plasmid DNA in 5% dextrose in water at room temperature. The *Y. pestis* F1 capsular antigen was purified from cultured *Y. pestis* strain CO92 and provided by Dr. Martin Schrieffer (CDEC, Fort Collins, CO). The F1 antigen was added to the pre-formed CLDC and mixed by gentle pipetting to form the final vaccine. The vaccine was administered within 30 min of preparation.

2.4. Immunizations

For all experiments, mice were immunized twice with the F1/CLDC vaccine, 2 weeks apart, and were challenged 4 or 18 weeks after the last immunization. Mice immunized orally received 10 µg F1 antigen ($n = 5$ per group) in a total volume of 400 µl of CLDC, administered by oral gavage. Mice immunized s.c. received 10 µg F1 antigen in a total volume of 200 µl of CLDC. Controls included mice immunized orally with 10 µg F1 antigen alone, or with CLDC adjuvant alone. All mice were vaccinated twice, 2 weeks apart. As indicated, vaccinated mice were challenged either 4 or 18 weeks after the last vaccination.

2.5. Determination of F1-specific antibody titers

Antibody titers to F1 in serum, saliva, and fecal pellets were determined using an ELISA. For determination of antibody titers in saliva, saliva was collected from anesthetized mice using pipet tips. For determination of fecal antibodies, fecal pellets were dissolved in (1 pellet in 1 ml PBS), then centrifuged and supernatants collected for assay. Briefly, Maxisorp microtiter plates (Nunc, Thermo-Fisher, Rochester, NY) coated overnight with F1 protein (5 µg/ml) diluted in 50 mM carbonate/bicarbonate buffer. After coating, wells were washed with PBS + 0.05% Tween 20 and blocked for 1 h at room temperature or overnight at 4 °C with PBS + 3% BSA. Plates were washed again and mouse serum, serially diluted in PBS + 1% BSA, was added and incubated for 90 min at room temperature. Plates were washed again and bound serum antibody was detected following addition of HRP conjugated goat anti-mouse IgA and IgG (Jackson ImmunoResearch, West Grove, PA) and addition of TMB substrate. Absorbance was measured at 450 nm using a Thermo Multiskan Ascent plate reader (Thermo-Fisher, Rochester, NY). Endpoint titers were determined as the last well greater than or equal to 0.1 O.D. units above the average of the blank wells.

2.6. Animal challenge

Mice were subjected to lethal i.n. challenge with a $100 \times \text{LD}_{50}$ dose of the MG05 strain of *Y. pestis*. Prior to challenge, mice were anesthetized by i.p. administration of a ketamine and

xylazine solution (Fort Dodge, Overland Park, KS). Bacteria were diluted in PBS and approximately 4×10^4 CFU in 25 μ l was delivered to the nares of each mouse. Actual inoculum concentration was confirmed by plating a portion of the inoculum onto BHI agar plates, incubating plates at 37 °C with 5% CO₂ and enumerating colonies. This challenge dose of *Y. pestis* routinely resulted in a mean time to death of 3 days in naïve C57Bl/6 mice. Following inoculation, mice were monitored closely and any ill mice that met pre-determined endpoints for euthanasia were immediately euthanized and these time points were used to calculate survival times. Mice that survived the challenge were monitored for survival for 30 days and were then humanely euthanized and organs plated to assure that *Y. pestis* could not be cultured.

2.7. Tissue collection and enumeration of bacteria

Lungs, livers spleens and blood of infected and control mice were collected 48 h after *Y. pestis* infection. Lungs, livers, and spleens were homogenized in sterile PBS using a stomacher (Teledyne Tekmar, Mason, OH). The resulting homogenate, or blood, was immediately serially diluted in PBS and plated on BHI agar and incubated at 37 °C for 24 h for enumeration of bacterial loads.

2.8. Statistical analyses

Statistical analyses were done using Prism v. 5.0 (GraphPad Software, La Jolla, CA). Kaplan–Meier survival curves and the log rank test was used to compare overall survival times. In Kaplan–Meier survival analyses involving comparisons of more than 2 groups, the Bonferroni corrected threshold was applied to determine the appropriate level of significance. For experiments involving comparison of 2 groups, the Mann–Whitney test was used for statistical analysis. Comparison of multiple treatment groups was performed using ANOVA, followed by Tukey's multiple means comparison test. A *p*-value of 0.05 or less was considered statistically significant for these analyses.

3. Results

3.1. Oral immunization with F1/CLDC vaccine elicits strong humoral immune responses

Protection against infection with virulent *Y. pestis* following parenteral vaccination is often associated with presence of F1-specific antibodies [8]. Thus, an effective oral immunization with F1/CLDC should generate titers of F1-specific antibody comparable to those observed following subcutaneous vaccination. To determine if oral immunization with the F1/CLDC vaccine was capable of eliciting such an immune response, we first assessed the development of F1-specific antibodies in the serum of vaccinated animals. Humoral immune responses to oral immunization with the F1/CLDC vaccine were also compared to those elicited by s.c. immunization with the F1/CLDC vaccine. Mice immunized orally with F1/CLDC developed significantly increased antibody titers against the F1 antigen, compared to unvaccinated control mice (Fig. 1A). Notably, the titer of the F1-specific IgG response elicited by oral immunization was equivalent to that elicited by s.c. immunization. As a control, we noted that oral immunization with F1 antigen alone did not elicit detectable F1 antibody responses (data not shown).

Mucosal immunization is often effective for eliciting IgA responses [34,35]. Therefore, we also determined the titer of F1-specific IgA present in the serum of vaccinated animals. Mice immunized orally with the F1/CLDC vaccine developed significant F1-specific IgA titers, comparable to the IgA titers elicited by s.c. immunization (Fig. 1B). Thus, oral immunization with the F1/CLDC vaccine generated significant increases in titers of IgG and IgA antibodies directed against F1, and titers were comparable to those elicited by s.c. immunization.

3.2. Oral immunization elicits effective protection against lethal pneumonic *Y. pestis* infection

Given the high titers of F1-specific antibodies generated in F1/CLDC vaccinated animals, we hypothesized that these animals would be protected against lethal pulmonary challenge with virulent *Y. pestis*. As expected, all unvaccinated control mice succumbed to infection within 3 days of challenge. Subcutaneous vaccination with F1/CLDC resulted in 90% protection against *Y. pestis* challenge (Fig. 2). Similarly, 80% of orally (p.o.) vaccinated mice were protected from lethal challenge (Fig. 2). Protection afforded by the F1/CLDC vaccine was dependent on the presence of F1 and CLDC since none of the mice treated orally or subcutaneously with CLDC adjuvant alone or orally with F1 without adjuvant were not protected from *Y. pestis* infection (data not shown). Thus, oral immunization with F1/CLDC was capable of generating protective immunity against pulmonary *Y. pestis* infection.

3.3. Oral immunization results in long-lived protection

The duration of protective immunity is also an important property of vaccines. Therefore, we examined whether the orally administered F1/CLDC vaccine was capable of eliciting longer lasting immunity. Mice were immunized twice p.o. or s.c. with F1/CLDC and then challenged with *Y. pestis* MG05 18 weeks after the last vaccination. We observed that the F1/CLDC vaccinated mice had a significant increase in survival compared to unvaccinated mice (Fig. 3). Moreover, similar percentages of surviving mice following the 4-week challenge were also observed in the 18-week vaccinated animals, with 80% of orally vaccinated animals and 100% of s.c. vaccinated mice surviving challenge (Fig. 3). These results indicated that oral immunization was capable of eliciting long-lived immunity to pulmonary *Y. pestis* infection and that this immunity did not significantly wane over time.

3.4. Oral immunization controls bacterial replication

To determine whether protection from *Y. pestis* challenge observed in F1/CLDC vaccinated mice was associated with direct control of bacterial replication and dissemination, we next assessed bacterial loads in specific target organs in vaccinated and naïve mice shortly after challenge. F1/CLDC vaccinated animals, regardless of route, had significantly fewer bacteria in the lung and liver compared to unvaccinated controls (Fig. 4). While bacterial loads in the spleens of vaccinated animals were numerically lower than those observed in naïve mice, these differences were not statistically significant (Fig. 4). Together these data indicated that immunization with F1/CLDC induced protective immune response in both mucosal sites (lung) and in peripheral tissues (liver, spleen) that were capable suppressing bacterial replication, but not completely blocking dissemination.

3.5. Role of CD4⁺ and CD8⁺ T cells in protective immunity to *Y. pestis* following oral immunization

Previous studies have shown that T cells are required for full vaccine-induced immunity to *Y. pestis* [36–38]. Therefore, we investigated whether CD4⁺ or CD8⁺ T cells were important in generating protection from *Y. pestis* challenge following immunization with F1/CLDC. Thus, wild type mice and CD4^{−/−} or CD8^{−/−} mice were vaccinated p.o. with F1/CLDC and then challenged with *Y. pestis*.

In p.o. vaccinated CD4^{−/−} mice, survival was significantly reduced ($p = 0.002$) compared to p.o. vaccinated wild type mice (Fig. 5). In p.o. vaccinated CD8^{−/−} mice challenged with *Y. pestis*, survival was also decreased compared to control mice, though the difference ($p = 0.11$) did not reach the level of statistical significance. When survival times for CD4^{−/−} versus CD8^{−/−} mice were compared statistically, the p -value ($p = 0.05$) did not reach the level of statistical significance with the Bonferroni correction applied. Thus, it was apparent

from these results that both CD4⁺ and CD8⁺ T cells played a role in mediating F1/CLDC induced protective immunity against virulent *Y. pestis*.

4. Discussion

Mucosal administration of vaccines offers a number of advantages over parenteral vaccination, including greater ease of administration and improved effectiveness for eliciting mucosal protective humoral and cellular immune responses [34,39–42]. Previously, oral delivery of protein antigens encapsulated in micro-spheres has been reported to generate effective humoral immunity [39,42–44]. However, at present only live attenuated vaccine vectors (e.g., *Salmonella* vectored vaccines) have been reported to be effective in eliciting protective immunity against inhaled pathogens such as *Y. pestis* [26,28,30,45–47]. Nonetheless, there remains a strong need for simple vaccine adjuvants capable of generating effective immunity following oral immunization, thereby avoiding the use of replicating vaccine vectors and the potential risk they entail.

In the present study, we found that effective protective immunity against lethal inhalational challenge with *Y. pestis* could be achieved by oral vaccination with *Y. pestis* F1 antigen combined with CLDC adjuvant. Notably, oral immunization with the F1/CLDC vaccine elicited IgG and IgA titers that were equivalent to those elicited by s.c. immunization. In addition, the humoral immune response to oral immunization with F1/CLDC was similar to that elicited by s.c. immunization with an F1/aluminum hydroxide vaccine (data not shown). Therefore, we concluded that CLDC could be used effectively as an oral vaccine adjuvant for generating antibody responses against protein antigens.

Protective immunity against *Y. pestis* generated by oral immunization in the present study was dependent on both humoral and cellular immune responses. For example, protection was associated with induction of high anti-F1 antibody titers and with rapid inhibition of bacterial replication in the lungs and livers of immunized mice. Consistent with a key role for antibodies in vaccine-induced protection, protective immunity was completely abrogated in CD4^{−/−} mice immunized orally with the F1/CLDC vaccine. This dependency on CD4 responses is similar to results reported recently using a VSV-vectored vaccine against *Y. pestis* [48]. Interestingly, full protection was also partially dependent on CD8⁺ T cells, since vaccinated CD8^{−/−} mice were only partially protected against lethal challenge. Thus, both subsets of T cells were required for full protection following oral vaccination with the CLDC-based vaccine.

Previous studies in mice vaccinated with live attenuated *Y. pestis* have also demonstrated a role for CD4⁺ and CD8⁺ T cells in protective immunity [37,49]. In addition, Kummer and Parent [38,50] have shown that protection can also be mediated by cytokines such as TNF- α and IFN- γ . In the present study, protection generated by oral immunization may be less dependent on cytokines such as IFN- γ , inasmuch as we did not observe IFN- γ production by T cells following re-stimulation of spleen or lung cells with F1 antigen in vitro (data not shown). However, we cannot exclude a role for other protective cytokines produced by CD4 T cells elicited by oral immunization.

In summary, we show that an effective orally delivered vaccine against *Y. pestis* can be developed using CLDC as a mucosal vaccine adjuvant. Additional studies will be required to more fully define relevant GI antigen presenting cells and the sites of antigen presentation for generating effective *Y. pestis* immunity following oral vaccination with this non-replicating vaccine. Taken together, these findings indicate that it may be possible to vaccinate orally using simply formulated vaccines based on CLDC adjuvants.

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Fig. 1.

Antibody responses to oral and s.c. immunization with F1/CLDC vaccine. Mice ($n = 5$ per group) were immunized twice, 2 weeks apart, with $10 \mu\text{g}$ *Y. pestis* F1 antigen complexed to CLDC adjuvant, either orally (p.o.) or s.c., as described in Section 2. Two weeks after the last immunization, blood was collected by tail vein and endpoint antibody titers in serum were determined, using an F1 ELISA. Mice immunized p.o. or s.c. had significantly increased IgG and IgA titers to F1 antigen compared to unvaccinated mice, as assessed by ANOVA. Titers were not significantly different between p.o. and s.c. vaccinated mice. Similar results were obtained in 2 additional experiments.

Fig. 2.

Oral immunization with F1/CLDC vaccine and protection against pulmonary *Y. pestis* infection. Mice ($n = 5$ per group) were immunized twice p.o. or s.c. with the F1/CLDC vaccine. Four weeks after the second immunization, mice were subjected to lethal i.n. challenge with a $100 \times \text{LD}_{50}$ dose of the MG05 strain of *Y. pestis*, as described in Section 2. Mice were euthanized when pre-determined disease endpoints were met and overall survival times were compared using Kaplan–Meier survival curves, followed by log rank test. Data presented represent pooled results from 2 independent experiments. Survival times were significantly increased ($p < 0.05$) in both p.o. and s.c. immunized mice, compared to unvaccinated control mice.

Fig. 3.

Protection against pulmonary *Y. pestis* infection 18 weeks after oral immunization. Mice ($n = 5$ per group) were immunized twice, 2 weeks apart, orally or s.c. with F1/CLDC vaccine. Eighteen weeks later, the mice were subjected to lethal i.n. challenge with 100 LD₅₀ *Y. pestis* MG05 strain. Mice were euthanized when predetermined disease endpoints were met and overall survival times were compared using Kaplan–Meier survival curves, followed by log rank test. Survival times were significantly ($p < 0.05$) longer in vaccinated mice compared to unvaccinated animals.

Fig. 4.

Bacterial burden in tissues of vaccinated and control mice after challenge with *Y. pestis*. Mice ($n = 4$ per group) were immunized twice, either p.o. or s.c., with F1/CLDC vaccine, as described in Section 2. The mice were then subjected to i.n. challenge with $100 \times \text{LD}_{50}$ of *Y. pestis* and euthanized 48 h later. Lung, liver, and spleen tissues from each mouse were homogenized and the bacterial CFU per organ was quantitated as described in Section 2. The mean bacterial counts per organ in vaccinated and control groups of animals were compared statistically using ANOVA and Tukey's multiple means comparison test. * denotes $p < 0.05$ compared to unvaccinated control animals. Similar results were obtained in 1 additional experiment.

Fig. 5.

Protection following oral immunization in wild type, CD4^{-/-}, and CD8^{-/-} mice. Wild type mice and CD4^{-/-} and CD8^{-/-} mice ($n = 5$ per group) were immunized twice orally with the F1/CLDC vaccine, then 4 weeks later were subjected to i.n. challenge with $100 \times \text{LD}_{50}$ *Y. pestis*. Mice were euthanized when pre-determined morbidity endpoints were reached and survival times were compared using Kaplan–Meier curves and log rank test, followed by the Bonferroni correction for multiple survival curve analysis. Survival times were significantly shorter ($p = 0.002$) in CD4^{-/-} mice than in wild type mice. For CD8^{-/-} mice, survival times were shorter than in wild type mice, but the difference did not reach the level of statistical significance ($p = 0.11$). When survival times in CD4^{-/-} and CD8^{-/-} mice were compared, the differences were not statistically significant ($p = 0.05$) when allowing for the Bonferroni correction. Data represent pooled results from 2 independent experiments.