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PRINCIPAL INVESTIGATOR: John D. Clements, Ph.D.

CONTRACTING ORGANIZATION: Tulane University Health Sciences Center
New Orleans, Louisiana 70112

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Enhancing the Immune Response to Recombinant Plague Antigens

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Author(s): John D. Clements, Ph.D.

Performing Organization: Tulane University Health Science Center
New Orleans, Louisiana 70112

E-Mail: jclemen@tulane.edu

Sponsoring Agency: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Abstract:
The etiologic agent of plague is the Gram negative bacterium Yersinia pestis. Y. pestis is a concern as one of the microorganisms with potential for use against civilian or military populations as a biological warfare/biological terrorism agent. In that case, the pneumonic form of plague would be the most likely outcome. This form of plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers. The studies reported herein explore the ability of a novel adjuvant, designated LT (R192G), to promote the rapid development of long-lasting, high titer antibodies against a recombinant plague antigen (F1-V) in a murine model. Subsequent studies will be performed in non-human primates. Different routes of administration are examined to test the hypothesis that heterologous boosting will be more effective than homologous boosting at increasing the magnitude and/or duration of the antibody response.

Subject Terms: Biological warfare, vaccine, adjuvant, immunization, plague, anthrax, Y. pestis

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Introduction

The etiologic agent of plague is the Gram-negative bacterium *Yersinia pestis*. *Y. pestis* is a concern as one of the microorganisms with potential for use against civilian or military populations as an agent of biological warfare or biological terrorism. In that case, the pneumonic form of plague would be the most likely outcome. This form of plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers. A number of potential subunit vaccine against plague have been evaluated for immunogenicity and protective efficacy. The two most promising are the *Y. pestis* proteins F1 and V. F1 is a capsular protein located on the surface of the bacterium and the V-antigen is a component of the Type III secretion system. In previous studies, combined immunization with native F1 and recombinant V (rV) in a two-dose regimen afforded full protection in mice against subcutaneous challenge with *Y. pestis* (8) and the anti-F1 and anti-V titers, especially of the IgG1 sub-class, correlated significantly with protection in BALB/c mice. Male and female CBA, C57/BL6 and CB6F1 mice were also protected against injected and aerosol challenge with *Y. pestis* following immunization with two doses of rF1 and rV (4). The combination or fusion of F1 and V has been has an additive protective effect in the murine model when compared to either antigen alone (1-3, 5-7). Heath et al. (3) reported construction of a F1-V fusion consisting of the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V-antigen. F1-V was shown to provide excellent protection against both subcutaneous and aerosol challenge and has the potential to provide protective immunity against pneumonic as well as bubonic plague due to either wild type F1+ *Y. pestis* or to naturally occurring F1- variants.

Soluble protein-based vaccines, such as F1-V, are generally administered subcutaneously or intramuscularly in the presence of an aluminum salt adjuvant. For most proteins, this is an effective means of inducing serum antibody against the antigen (i.e., tetanus and diphtheria toxoid). Recently, a great deal of attention has been directed towards needle-free immunization strategies as alternative methods for vaccine delivery. Both mucosal (intranasal, oral, rectal) and transcutaneous immunization in the presence of an appropriate adjuvant have been shown to induce humoral and cellular immune response in both the systemic and mucosal compartments. Alternating routes for delivery of the priming dose and booster dose in immunizations, so called “prime-boost” strategies have also been examined for the ability to induce high-titer, long-lasting humoral responses and have the potential direct or redirect the immune response to one compartment or another. This may be particularly useful for development of vaccines against agents that may be delivered by aerosol, where the respiratory mucosa would be the first point of productive contact between the organism and the host.

In the current study, we examine different prime/boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the ability of recombinant F1-V to promote the development of long-lasting, high titer antibodies. We also examine the effect of different prime/boost regimes on the compartmentalization of the ensuing immune response. For parenteral immunization, F1-V is adsorbed to aluminum hydroxide, which is commonly used as an adjuvant for parenterally administered vaccines. Mucosally and transcutaneously administered vaccines are usually not immunogenic and also require the presence of an appropriate adjuvant. In the studies reported here, we utilize a mutant of the heat-labile enterotoxin of *Escherichia coli,*
designated LT(R192G), that has been shown to be effective when administered mucosally (orally, rectally, intranasally) or transcutaneously in a variety of animal models and in humans.

Body

This project is organized into two Specific Aims that constitute the Technical Objectives of the proposal.

**Specific Aim 1. Optimize the Immune Response to F1-V in a Murine Model.** In the first specific aim, we examine the ability of LT(R192G) to function as an adjuvant for F1-V when delivered mucosally or transcutaneously and the ability of adjuvanted mucosal or transcutaneous immunization to serve as a booster for parenteral priming. The primary objective of this aim is to optimize immunization to achieve a rapid anti-F1-V antibody response of high titer and of long duration. Another objective of this aim is to determine if the antibody response to both antigens, F1 and V, is sustained. The optimum prime/boost regimen from these studies, as defined by antibody responses and confirmed by challenge, will subsequently be examined in Non-Human Primates (NHP).

**Specific Aim 2. Evaluate the Immune Response to F1-V in Non-Human Primates.** The second specific aim is to evaluate the optimum prime/boost regimen from Specific Aim 1 in nonhuman primates. The primary objective of this aim is to optimize immunization to achieve a rapid response of high titer and of long duration. Another objective of this aim is to determine if the antibody response to both antigens, F1 and V, is sustained. Animals will be followed monthly to determine their antibody response to parenteral F1-V and whether the optimum prime/boost regimen from Specific Aim 1 can enhance and extend that response. Since previous studies have suggested that F1-V administered parenterally is not optimally protective in NHP (Dr. Jeffrey Adamovicz, Personal Communication), immunized animals will be transferred to a USAMRIID designated facility for challenge.

During this reporting period cover, we successfully immunized all animals as specified in the approved Scope of Work for Specific Aim 1 and analyzed the serum and BAL responses through the twelve-months post-primary immunization. Data from two, six and twelve-months are shown below. Groups of 6-8 week old female Swiss Webster mice (Charles River Laboratories) were immunized twice (day 0 and day 28) with recombinant F1-V. Mice were immunized subcutaneously (SC), intranasally (IN), or transcutaneously (TCI) and then boosted by the same (homologous) or a different (heterologous) route as shown in the following table.

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<thead>
<tr>
<th>Priming Dose</th>
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Mice immunized subcutaneously received 10 μg of recombinant F1-V adsorbed to an aluminum hydroxide adjuvant (2.0% Alhydrogel batch no. 3275; Superfos Biosector, Vedbaek, Denmark) in a final volume of 100 μl. Mice immunized intranasally received 5 μg of recombinant F1-V admixed with 5 μg LT(R192G) in a final volume of 9.6 μl in one nostril following brief exposure to Isoflurane. Mice immunized transcutaneously received 35 μg F1-V admixed with 25 μg LT(R192G) in a final volume of 50 μl applied to freshly shaved dorsal skin following intraperitoneal injection of ketamine.

**Measurement of serum and bronchioalveolar lavage antibody.** Mice were sacrificed in groups of 5 from each of the 3 primary immunization groups at monthly intervals. Blood was obtained from each animal by cardiac puncture. Lung lavage fluid was collected from each animal by exposing the trachea, making a small incision, inserting and securing an 18-gauge needle, and aspirating 1 ml of PBS three times before final withdrawal. Serum and bronchioalveolar lavage (BAL) fluid were examined for the presence of anti-F1-V by ELISA. Briefly, ELISA plates were coated with 0.1 μg per well of recombinant F1-V in 100 μl ammonium bicarbonate buffer. Following overnight incubation at 4°C, plates were washed in PBS containing 0.3% Tween 20, and two-fold serial dilutions of the samples were applied. After incubation for 1 hour at room temperature, plates were washed and a 1:400 dilution of goat anti-mouse IgG1 labeled with alkaline-phosphatase was added and incubation continued for 1 hour at room temperature. Plates were washed and the substrate PNPP was added. The reaction was stopped with 2N NaOH and the plates were read at an optical density at 405 nm. Concentration was determined by nonlinear regression against a standard curve of mouse monoclonal IgG1. The results are expressed as the mean concentration ± SEM.

**Statistical Analysis.** Values for the mean and standard error of the mean were calculated for each group for each time point. Statistical analyses were performed by using a one-way analysis of variance with Bonferroni’s Multiple Comparison post-test.

**RESULTS**

**Homologous prime-boost.** The purpose of this group of experiments to was compare three different routes of immunization (intranasal, transcutaneous, subcutaneous) for the ability to induce high titer anti-F1-V serum and BAL antibody responses following one or two immunizations with the priming dose and booster dose delivered by the same (homologous) route. Mice were immunized once or twice with F1-V adsorbed with alum (SC) or admixed with LT(R192G) (IN, TCI) and groups of five animals from each regime were sacrificed periodically over twelve-months. Data from two, six and twelve-months are shown.

A single primary immunization with F1-V by any of the three routes induced only a minimal anti-F1-V serum or BAL antibody response by 28 days post-primary immunization. The responses to a single immunizing dose did not rise over time (data not shown).

Following a single homologous boosting dose administered at day 28, serum and BAL anti-F1-V responses increased in all three groups with peak responses observed at day 59 (month 2) (see Figure 1). The apparent increases from month 2 through month 12 for TCI serum (Fig. 1.B) and BAL (Fig. 1.E) are not statistically significant (p>.05) when compared to the serum and BAL anti-F1-V IgG1 response at month 2. The highest serum and BAL anti-F1-V responses were
obtained following IN and SC immunization, which were not different from one another at any time point over the twelve-month period (p>.05). Both IN and SC regimes induced significantly higher levels of serum and BAL anti-F1-V IgG1 than did transcutaneous immunization with the same antigen (p<.05). Taken together, these results demonstrate that intranasal and subcutaneous immunization are essentially equivalent for induction of serum and BAL anti-F1-V IgG1 responses when a single booster dose is administered by the same (homologous) route.

**Heterologous prime-boost.** Heterologous boosting offers the possibility of increasing the magnitude or duration of the immune response when compared to homologous boosting and may also influence the compartmentalization of that response. For these studies, animals received a primary immunization by one route and then a single booster dose by an alternate route (Table 1). As above, groups of five animals from each regime were sacrificed periodically over twelve-months. Data from two, six and twelve-months are shown.

As seen in Figure 2.A, animals primed IN and then boosted SC developed higher levels of serum anti-F1-V than did animals primed IN and boosted either IN or TCI (p<.01). This response remained elevated through six-months and declined thereafter. The serum anti-F1-V responses in animals primed IN and boosted IN or TCI remained elevated through six-months, although clearly at a lower level than that obtained with IN priming and SC boosting. By contrast, either IN priming followed by IN or TCI boosting or SC priming followed by TCI boosting produced the highest level of BAL anti-F-V. These responses were maximal at two-months post-primary immunization and declined significantly by six-months.

These findings demonstrate that heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses. Specifically, animals primed by one route (e.g., SC) can be boosted by the same (SC) or a different (IN, TCI) route with no significant diminution of the serum or BAL anti-F1-V IgG1 response. In no case was heterologous boosting inferior to homologous boosting and in three specific cases (IN x SC serum anti-F1-V; IN x TCI BAL anti-F1-V; SC x TCI BAL anti-F1-V) heterologous boosting was more effective than homologous boosting.

**Effect of priming on the compartmentalization of the immune response.** Since *Y. pestis* and other microorganisms that represent a significant biological warfare threat may be spread by the aerosol route, a primary consideration for an effective vaccine may be the ability to induce an effective immune response at the level of the bronchoalveolar surface. Data presented in Figures 1 and 2 suggest that IN and SC priming may be equivalent for induction of a BAL anti-F1-V IgG1 response. To further examine this issue, a comparison was made between animals immunized IN, TCI, or SC and then boosted by any route (i.e., IN prime followed by IN, TCI, or SC boost).

As seen in Figure 3.A, IN and SC priming were more effective than TCI priming for induction of serum anti-F1-V IgG1 when the boost was administered by any route (p<.01) and not different from one another (p>.05) through six-months post-primary immunization. By six-months post-primary immunization, the serum anti-F1-V IgG1 response following SC prime and boost by any route was significantly higher than that following with IN prime (p<.01) or TCI prime (p<.001) with the same antigen. With respect to BAL responses (Fig. 3.B), either IN or SC prime followed by any boosting route induced significantly higher BAL anti-F1-V IgG1 than TCI priming.
(p<.001), at least through six-months post-primary immunization, clearly demonstrating that either IN or SC priming may be effective when a bronchialveolar response is desired.

Key Research Accomplishments

- Intransal and subcutaneous immunization are essentially equivalent for induction of serum and BAL anti-F1-V IgG1 responses when a single booster dose is administered by the same (homologous) route.

- Heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses.

- In no case was heterologous boosting inferior to homologous boosting and in three specific cases heterologous boosting was more effective than homologous boosting.

- IN and SC priming were more effective than TCI priming for induction of serum anti-F1-V IgG1 when the boost was administered by any route and not different from one another through six-months post-primary immunization.

- With respect to BAL responses, either IN or SC prime followed by any boosting route induced significantly higher BAL anti-F1-V IgG1 than TCI priming, at least through six-months post-primary immunization, clearly demonstrating that either IN or SC priming may be effective when a bronchialveolar response is desired.

Reportable Outcomes – None to Date

Conclusions

In the current study, we examine different prime/boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the ability of recombinant F1-V to promote the development of long-lasting, high titer antibodies. We also examined the effect of different prime/boost regimes on the compartmentalization of the ensuing immune response. The most significant finding was that heterologous boosting can be as or more effective for induction of serum and/or BAL responses than homologous boosting. The ability to administer a mucosal or transcutaneous booster following parenteral priming would, in itself, revolutionize the administration of vaccines. It was also notable that both IN and SC priming were effective at inducing BAL responses, which may partially explain why animals immunized with F1-V parenterally were protected against aerosol challenge. It is likely that the anti-F1-V antibody detected in the BAL from immunized animals was transudated from the serum and not specifically secreted (the lung is not an efficient secretory organ).

It remains to be determined if protection against aerosolized Y. pestis will correlate with anti-F1-V serum or BAL antibody responses. Those challenge studies are scheduled to be conducted at USAMRIID during the second quarter of 2004. Animals for those challenge studies have been immunized and are awaiting transport. Non-human primate studies outlined in Specific Aim 2 will follow, the exact composition of which will be determined following the murine challenge studies.
References


**Figure 1. Homologous prime-boost.** Mice were immunized with F1-V adsorbed with alum (SC) or admixed with LT(R192G) (IN, TCI) and groups of five animals from each regime were sacrificed monthly over twelve-months. Serum and BAL anti-F1-V IgG1 concentrations were determined by ELISA. Bars represent mean serum anti-F1-V IgG1 (μg/ml) (A, B, C) or BAL anti-F1-V IgG1 (ng/ml) (D, E, F) + SEM. Data from two-, six- and twelve-months are shown. 1.A and 1.D: Groups of mice received a primary IN immunization followed by an IN boost (black bars). 1.B and 1.E: Groups of mice received a primary TCI immunization followed by a TCI boost (stippled bars). 1.C and 1.F: Groups of mice received a primary SC immunization followed by an SC boost (white bars).
Figure 2. Heterologous prime-boost. Mice were immunized with F1-V adsorbed with alum (SC) or admixed with LT(R192G) (IN, TCI) and groups of five animals from each regime were sacrificed monthly over twelve-months. Serum and BAL anti-F1-V IgG1 concentrations were determined by ELISA. Bars represent mean serum anti-F1-V IgG1 (µg/ml) (A, B, C) or BAL anti-F1-V IgG1 (ng/ml) (D, E, F) + SEM. Data from two-, six- and twelve-months are shown. 1.A and 1.D: Groups of mice received a primary IN immunization followed by an IN (black bars), TCI (stippled bars) or SC (white bars) boost. 1.B and 1.E: Groups of mice received a primary TCI immunization followed by an IN (black bars), TCI (stippled bars) or SC (white bars) boost. 1.C and 1.F: Groups of mice received a primary SC immunization followed by an IN (black bars), TCI (stippled bars) or SC (white bars) boost.
Figure 3. Effect of priming on the compartmentalization of the immune response. Data presented in Figures 1 and 2 suggest that IN and SC priming may be equivalent for induction of a BAL anti-F1-V IgG1 response. To further examine this issue, a comparison was made between animals immunized IN (black bars), TCI (stippled bars), or SC (white bars) and then boosted by any route (i.e., IN prime followed by IN, TCI, or SC boost).