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14. 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) and protection 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.

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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* (15) 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 (6). 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 (3-5, 11, 13, 14). Heath et al. (5) reported construction of a an 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 contract, 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 current studies, 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. Aerosol challenge of immunized mice will be conducted to correlate the induction of serum and mucosal antibodies with protection. 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).

Status of Specific Aim 1. All of the requirement of this aim were completed at the time of the last report filing. The findings from these studies have been published in two manuscripts (see below) and the data will be used to down-select candidate vaccine approaches for Specific Aim 2.

Specific Aim 2 (Revised). Evaluate the Immune Response to F1-V in Non-Human Primates. The objective of this aim is to test the comparative efficacy of USAMRIID's current F1-V plague vaccine candidate with or without mucosal adjuvant administered in heterologous vaccination schemes against aerosol challenge in nonhuman primates. The candidate heterologous vaccination approaches will be down-selected from the mouse model of plague (Specific Aim 1) prior to testing in NHP. We hypothesize that heterologous prime/boost with rF1-V in conjunction with the mucosal adjuvant LT(R192G) or Alhydrogel will be superior to homologous route vaccination with rF1-V in Alhydrogel.

Status of Specific Aim 2. The original Scope of Work and timetable projected completion of Specific Aim 1 by the end of the first calendar year and completion of Specific Aim 2 by the end of the second calendar year. A number of obstacles prevented the timely initiation of Specific Aim 2. The first was a source of antigen, F1-V. There have been increased demands on limited supplies and we were not always able to obtain sufficient quantities of this antigen from USAMRIID in a timely manner. One provision of the contract was that USAMRIID had the option of requiring a mouse challenge study before proceeding to the NHP studies. At USAMRIID's request, animals to be challenged were immunized at Tulane University and shipped to USAMRIID for challenge. There was some delay while the composition of the challenge groups was agreed upon and an additional delay waiting for a window of opportunity to open at USAMRIID. A no-cost extension was granted to allow these studies to continue. Those studies were completed and reported in the last progress report. Subsequent to that mouse challenge study, USAMRIID investigators indicated a desire to expand the number of animals in the NHP study from the budgeted 8 to a total or 36, employ telemetry (requiring surgical manipulation of each animal), and charge the contract for the additional animals, telemetry

devices, surgery, and challenge (not part of the original contract). The original budget was based on 8 animals to be immunized at the Tulane National Primate Research Center and shipped to USAMRIID where challenge would be conducted for no charge, so there was simply no way to cover the additional costs within the existing budget. Consequently, a decision was made to move that portion of the study to USAMRIID and to use the balance of the funds at Tulane ($120,875) to reimburse USAMRIID to partially offset the cost of the expanded study. A CRDA (USAMRIID Control No. W81XWH-06-0270) formalizing the revised study plan and reimbursement was instituted and became effective on July 7, 2006 (ending date July 7, 2008).

An additional delay occurred due to the disruption of operations associated with Hurricane Katrina. All operations at Tulane University were effectively shut-down between August 29, 2005 and January 1, 2006.

KEY RESEARCH ACCOMPLISHMENTS

- 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 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 bronchioalveolar response is desired.

- As single SC immunization with F1-V alone, with or without alum as an adjuvant, was sufficient to protect mice against aerosol challenge with 70 LD50 of *Y. pestis* CO92.

- IN prime and boost with LT(R192G) as an adjuvant provided solid (100%) protection against aerosol challenge with 70 LD50 of *Y. pestis* CO92.

REPORTABLE OUTCOMES

Glynn A, Freytag LC, Clements JD. 2005. Effect of homologous and heterologous prime-boost on the immune response to recombinant plague antigens. Vaccine 23:1957-1965.

Glynn, A., C. J. Roy, B. S. Powell, J. J. Adamovicz, L. C. Freytag, and J. D. Clements. 2005. Protection against aerosolized *Yersinia pestis* challenge following homologous and heterologous prime-boost with recombinant plague antigens. Infect. Immun. 73:5256-5261.

Abstracts: Glynn, A, Freytag, L.C., and Clements, J.D. 2004. Enhancing the immune response to recombinant plague antigens. Presented at the 104th Annual Meeting of the American Society for Microbiology, New Orleans, LA

CONCLUSIONS

In the current study, we examined different prime - boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the effect of changing the route of prime and boost on 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 findings of the immunization study are that 1) IN and SC immunizations are both effective and 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, 2) heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses, and 3) anti-F1 and anti-V total IgG responses were highest in animals primed IN and boosted by any route when compared to animals primed TC or SC. As with previously published studies, there were still detectable levels of circulating anti-F1-V antibodies even one year post-primary immunization.

The observation that heterologous boosting may, in some cases, produce higher and more sustained antibody responses than homologous boosting is consistent with other reports that have examined this question (1, 7-10, 12). Most recently, Lauterslager et al. (7) demonstrated that oral (PO) boosting with ovalbumin was more effective in animals primed IN, SC, or intraperitoneally than PO (homologous) boosting with the same antigen. Similarly, Nicholas et al. (9) demonstrated that SC immunization was effective for priming animals subsequently boosted SC or IN (but not PO) with chimeric virus particles expressing a 17-mer peptide sequence from canine parvovirus (CPMV). In those studies, animals primed IN and boosted SC developed significantly higher serum anti-CPMV IgG2a responses than did animals primed IN and boosted IN. In the study of Baca-Estrada et al. (1) mice immunized SC with formalin killed whole cells and then boosted IN with formalin killed whole cells, either alone or formulated in liposomes, developed higher serum and BAL anti-*Y. pestis* antibody and higher systemic cell-mediated immune responses than did animals boosted SC. Our findings are in agreement with these studies, all of which demonstrate that heterologous boosting can be as or more effective that homologous boosting for induction of serum antibodies. Since serum IgG1 has been shown to be protective against aerosolized *Y. pestis*, mucosal IgA was not examined in the current study. However, Baca-Estrada et al. (1), Lauterslager et al. (7), and Nicholas et al. (9) each demonstrated that heterologous boosting could also induce significant mucosal IgA responses.

The fact that IN and TC boosting of SC-primed animals generated higher levels of anti-F1-V antibodies than homologous SC boosting is interesting and could be explained by the distribution of T effector-memory cells to the peripheral tissues following SC priming where they would be available to interact with cognate antigen applied mucosally or transcutaneously in the context of an appropriate adjuvant (e.g., LT(R192G)) (2). However, SC boosting of IN and TC primed animals was also more effective than homologous IN or TC boosting for induction of serum ati-F1-V antibodies, suggesting that a more global immunological phenomenon may be functioning

here. Moreover, the adjuvant employed for IN and TC immunizations may also play a role. A number of studies have shown that the ADP-ribosylating enterotoxins can induce phenotypic and functional maturation of dendritic cells as well as interacting directly with T-helper cells, B-cells, and epithelial cells. Both the Lauterslager et al. (7) and Nicholas et al. (9) utilized cholera toxin as a mucosal adjuvant. Clearly, the role of the adjuvant in controlling these outcomes requires further investigation.

The amount of antigen delivered by each route may also contribute to the outcome. Thus, the observation that both IN and SC regimens induced significantly higher levels of serum and BAL anti-F1-V IgG1 than did TC immunization with the same antigen (Fig. 2) may be influenced by the amount of antigen delivered. It is possible that the responses to TC prime and boost would be higher if larger amounts of antigen were applied or other methods were used to make uptake of transcutaneously administered antigens more efficient.

In and of itself, the observation that immunization by one route can prime for a secondary response by another route is important. In practical terms alone, especially in an imminent or post-release bioterrorism event, the ability to administer a parenteral priming dose and, at the same time, distribute a follow-on patch, pill, or nasal applicator that could be self-administered would greatly improve national preparedness.

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