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Current challenges in the development of vaccines for pneumonic plague

Stephen T Smiley, PhD

Associate Member, Trudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983, USA

Abstract

Inhalation of *Yersinia pestis* bacilli causes pneumonic plague, a rapidly progressing and exceptionally virulent disease. Extensively antibiotic-resistant *Y. pestis* strains exist and we currently lack a safe and effective pneumonic plague vaccine. These facts raise concern that *Y. pestis* may be exploited as a bioweapon. Here, I review the history and status of plague vaccine research and advocate that pneumonic plague vaccines should strive to prime both humoral and cellular immunity.

Keywords

cellular immunity; humoral immunity; pneumonic plague; *Yersinia pestis*

Bubonic, septicemic & pneumonic plague

Plague is an exceptionally virulent, zoonotic infection transmitted naturally from rodent reservoirs to humans via fleas [1,2]. *Yersinia pestis*, the disease-causing agent, was first identified by Alexander Yersin in 1894 [3]. This Gram-negative, nonmotile bacterium evolved recently from *Yersinia pseudotuberculosis*, an enteropathogen [2,4]. Evolution selected for traits that enable *Y. pestis* to achieve high titers within the mammalian bloodstream, thereby facilitating vector-borne transmission [5,6]. The spread of *Y. pestis* is also facilitated by the death of its mammalian hosts, which compels infected fleas to seek new hosts. In endemic areas, plague epizootics periodically decimate susceptible rodent populations. Humans are incidental victims in these deadly cycles.

Flea-borne human infections usually cause bubonic plague. The disease-defining symptom is the bubo, a painful swelling of the lymph nodes draining the fleabite. Without prompt antibiotic treatment, approximately 50% of bubonic plague cases progress to sepsis and death. Up to 30% of fleabites lead directly to sepsis, without prior evidence of a bubo [1,7].

Occasionally, bubonic and septicemic infections progress to secondary pneumonic infections. Pneumonic plague allows for direct person-to-person transmission via infectious respiratory droplets. The course of plague in individuals infected directly by airborne *Y. pestis* is even more virulent than that which ensues after fleabite [1,8,9]. Following an incubation period of 1–6 days, primary pneumonic plague develops very rapidly. Symptoms begin with rigor, severe headache, nausea and malaise. They quickly advance to fever, cough and difficulty breathing. The cough becomes increasingly productive, eventually yielding frothy, infectious, bright red sputum teeming with bacilli. Deaths result from respiratory failure and/or sequelae of severe sepsis, including circulatory collapse, coagulopathy and hemorrhage. Pneumonic plague is nearly always fatal unless treated with antibiotics within 20 h of symptom onset [1].

Pathology of plague

The pathology of bubonic plague is very similar in rodents, nonhuman primates and humans [1,10]. In a rat model of bubonic plague, *Y. pestis* bacilli initially accumulate in lymph nodes draining the site of intradermal injection [11]. The bacteria then multiply to extraordinarily high levels. The lymph nodes swell and become hemorrhagic, thrombotic and necrotic. In comparison with other bacterial infections, the host immune response is slow to respond. Despite the steadily growing bacterial burden, neutrophils fail to accumulate in large numbers. Macrophages begin to accumulate but their numbers soon decline, coincident with increased evidence of cellular apoptosis. In parallel, bacteria escape the node, access the bloodstream and spread to other tissues.

To the extent that information is available, rodent and nonhuman primate models of pneumonic plague also closely resemble the human disease. Mice infected intranasally display steadily progressive bacterial growth in pulmonary tissues, with dissemination to the spleen by 36 h post infection [12,13]. Within 4 days, they succumb to a purulent multifocal exudative bronchopneumonia accompanied by bacteremia, fibrin deposition, hemorrhage and necrosis. As for bubonic plague, the immune system appears slow to respond. The lungs display little evidence of inflammation at 24 h post infection. Only later do cytokine levels and neutrophil numbers increase. Rhesus macaques infected with aerosolized *Y. pestis* likewise develop a rapid onset pneumonia with bacterial dissemination to draining lymph nodes, spleen, liver and kidney, accompanied by fibrin thrombi, hemorrhage and necrosis [14,15]. Again, the robust neutrophil responses that typically characterize bacterial pneumonia are delayed and ineffective [15]. Human cases of pneumonic plague are uncommon today, but a comprehensive review by Wu Lien-Teh provides a detailed historical account of early 20th Century autopsy findings [8]. He noted little evidence of phagocytosis by leukocytes or other host defensive actions and stated, 'In no other form of pneumonia are the pathogenic bacteria to be found in such abundance... Considering the acute and quickly developing nature of the pulmonary process, the surprising thing is not that so many patients die but that even a few recover' [8].

Pathogenesis, lifestyle & virulence mechanisms

Y. pestis gene-expression patterns are regulated by temperature and other environmental variables [5,6]. Thus, the mammalian immune system encounters one form of *Y. pestis* upon fleabite transmission - where the infecting bacteria have grown at or near ambient temperature within the insect's midgut and a very different form of *Y. pestis* upon person-to-person transmission - where the bacteria have grown at 37°C within the human lung. When considering virulence, the route of infection is also an important criteria since a number of mutant *Y. pestis* strains are highly attenuated when administered subcutaneously, to mimic the fleabite, but retain substantially greater virulence when administered intravenously [16-19] or via pulmonary routes [20-22]. These distinctions highlight the need to characterize the host response to each form and route of *Y. pestis* exposure.

It is now widely accepted that the extreme virulence of *Y. pestis* results, to a large extent, from the host's failure to mount an adequate innate immune response [5,6,23-25]. Thus, modern-day plague vaccine researchers are focused, largely, on targeting *Y. pestis* virulence factors that impair innate immunity. Much remains to be learned about the mechanisms that enable *Y. pestis* to overwhelm host defense but a number of virulence factors have been identified and studied extensively. Here, I discuss general issues pertaining to virulence and introduce the *Y. pestis* proteins receiving greatest attention as targets for subunit vaccines. For detailed discussions on the fascinating biology of *Y. pestis* virulence factors, interested readers are referred to a series of excellent reviews [5,6,23-25].

Phagocytes are the innate immune system's primary defenders against acutely virulent extracellular bacteria. Researchers have long appreciated that *Y. pestis* replicates extracellularly and appears to do so unimpeded by phagocytes. While early research established that serum isolated from plague convalescents could passively transfer protection to naive mice, in 1944, Jawetz and Meyer concluded, 'serum, plasma, or other body fluids of animals immune to plague infection are unable to destroy or lyse [*Y. pestis*] organisms *in vitro* and *in vivo* in the absence of phagocytic cells' [26]. Meyer subsequently studied phagocytes at the site of intradermal *Y. pestis* infection in naive and immune monkeys. He observed pronounced phagocytosis in immune animals, beginning within 4 h, whereas phagocytosis was 'completely paralyzed' in naive animals [27]. He also demonstrated that immune serum has specific opsonizing activity but concluded, 'phagocytosis is the most important mechanism which animals and man use in guarding against and disposing of a plague infection' [27].

Initially, this capacity to resist phagocyte-mediated destruction was attributed to the gel-like capsule that surrounds *Y. pestis* bacilli grown at 37°C [28-32]. However, the capsule's primary constituent - the F1 protein - is dispensable for virulence in mice, primates and humans [33-36]. Nevertheless, there is ample evidence that growth at 37°C activates *Y. pestis* virulence mechanisms that impair phagocytic responses. Paramount among these is a plasmid-encoded, type III secretion system [5,6,23-25]. This system produces an 'injectisome' that translocates *Yersinia* outer proteins (Yops) to neighboring host cells, where they disrupt signaling pathways, prevent cytoskeletal rearrangements, suppress cytokine production, promote apoptosis and altogether debilitate the antibacterial defense mechanisms of phagocytes. *In vivo*, the Yops primarily target dendritic cells, macrophages and neutrophils [37]. Delivery of the Yops via the type III secretion system is functionally dependent on one of its substrates, low-calcium-response (Lcr)V. Several of the Yops, and LcrV, are critical for virulence, suggesting that deliberate impairment of phagocytic activity by the injectisome is one means by which *Y. pestis* evades host immunity [5,6,23-25].

Independent of the Yops, LcrV may directly contribute to the delayed inflammatory responses that characterize *Y. pestis* infection. First, purified LcrV inhibits neutrophil chemotaxis *in vitro* [38]. Second, purified LcrV can activate Toll-like receptor (TLR)2-mediated production of anti-inflammatory IL-10 [6,25]. It is notable, however, that the *Y. pestis* LcrV is a less efficient activator of TLR2-mediated IL-10 production than is *Y. enterocolitica* LcrV [39-41] and the relative importance of *Y. pestis* LcrV as a mediator of Yop translocation versus stand-alone, anti-inflammatory agents remains to be established decisively. Regardless, it is clear that LcrV is a key factor enabling *Y. pestis* to grow to high titers in mammals.

Y. pestis undoubtedly replicates extracellularly but the extent to which virulence relies upon intracellular replication remains a subject of considerable debate. *In vitro*, *Y. pestis* replicates within macrophages [30,42-44]. Nevertheless, detailed kinetic studies of mice infected intranasally [12] and rats infected intradermally [11], failed to observe significant numbers of intracellular organisms *in vivo*. However, a flow cytometry-based study readily detected *Y. pestis* within spleen cells of mice infected subcutaneously and, until the final days of infection, the splenic bacilli were found, almost exclusively, within CD11b-expressing macrophages [45]. Moreover, multiple studies of pneumonic plague in nonhuman primates have documented intact *Y. pestis* organisms within alveolar macrophages [15,36]. In an electron microscopy study of aerosol-infected macaques, Finegold observed 'many alveolar macrophages containing intact bacilli' and noted, 'the outcome of phagocytosis of the bacilli was generally unfavorable to the monocyte' with 'few examples of morphological damage to bacilli' [15].

Although the significance of intracellular bacteria has yet to be demonstrated decisively, altogether, the available data suggest that *Y. pestis* growing within phagocytes plays an important pathogenic role. Extracellular bacilli dominate the late stages of infection, but

intracellular organisms have even been detected at that time [15,36,45]. There seems to be growing consensus that cells of the monocyte/macrophage lineage offer *Y. pestis* a protected intracellular niche that provides time to adjust to growth within mammals [30], in part by upregulating expression of capsular F1 protein, LcrV and Yops, and thus enabling subsequent growth as extracellular, phagocyte-resistant bacilli.

Plague as a weapon

Pandemics of plague have afflicted human civilizations throughout recorded history and isolated outbreaks continue to this day in many regions of the world [2,5,46]. Most outbreaks bring primarily bubonic disease, but occasionally they contain high frequencies of pneumonic disease and person-to-person transmission [8,47,48]. Fortunately, simple gauze cotton masks effectively prevent person-to-person transmission [8,9]. Moreover, improved sanitation and public health surveillance, coupled with effective antibiotics and a better understanding of transmissibility, greatly reduce the likelihood of a modern day pandemic [1,2,9].

Recent interest in plague stems largely from bioterrorism concerns [2,46]. Indeed, plague has a long history as an agent of biowarfare [2,46,49,50]. It is thought that in 1347, the Tartars catapulted plague-ridden corpses into the besieged city of Kaffa, causing residents to flee and spread the Black Death to Italy. During World War II, the Japanese dropped ceramic bomblets containing *Y. pestis*-infected fleas on Chinese cities, initiating local outbreaks of bubonic plague. During the Cold War, both American and Soviet scientists devised means to effectively aerosolize *Y. pestis*, thereby removing the need for the flea vector. Remarkably, small cities in the Soviet Union were largely devoted to the production of weapons-grade infectious agents, including *Y. pestis*, suitable for deployment via intercontinental missiles [49]. One of today's primary concerns is that rogue scientists from the Cold War era may be willing to share the knowledge required to produce and deploy aerosolized *Y. pestis* [2,49]. In addition, antibiotic-resistant *Y. pestis* strains are now known to exist [51-53]. Covertly aerosolized, antibiotic-resistant *Y. pestis* would be a formidable bioweapon [46].

First plague vaccines

A safe and effective pneumonic plague vaccine would thwart the use of *Y. pestis* as an agent of terror. Unfortunately, over 100 years of research have yet to generate a safe and effective pneumonic plague vaccine [54]. Haffkine described the first widely used vaccine in 1897 [55,56]. He injected vaccine recipients with a heat-killed broth of densely grown, fully virulent *Y. pestis* organisms [55]. Although controlled trials were not performed, Haffkine's vaccine likely conferred humans with significant protection against bubonic plague [56]. However, the recommended dosage was meant to produce a fever of 102°F in the majority of human recipients [56], and severe adverse reactions limited its acceptance [57]. Moreover, experimental studies in rodents and nonhuman primates confirmed efficacy against bubonic disease, but generally failed to demonstrate protection against pneumonic disease [8].

In the mid-20th Century, Meyer and colleagues championed the development of more refined whole-cell plague vaccines comprised of formalin-killed *Y. pestis* organisms suspended in saline solution [57,58]. Ultimately, a vaccine of this type was licensed and sold as Plague Vaccine, USP. Controlled clinical trials were not performed, but there is strong evidence that formalin-killed whole-cell vaccines protected US military personnel against bubonic plague during the Vietnam War [58,59]. However, these vaccines also cause significant adverse reactions, including fever, headache, malaise, lymphadenopathy, erythema and induration at the injection site [1,57]. Adverse reactions are particularly prominent during booster injections, which are needed to maintain protection [1]. Accordingly, enthusiasm for formalin-killed vaccines gradually waned. In addition, they generally failed to protect mice and nonhuman primates against pulmonary *Y. pestis* challenge, and several humans contracted pneumonic

plague despite prior vaccination [54,57,58,60]. Thus, formalin-killed whole-cell vaccines are not deemed suitable for defense against weaponized pneumonic plague.

In 1904, Kolle and Otto protected experimental rodents against plague by vaccinating them with relatively small quantities of live-attenuated *Y. pestis* bacilli [61]. Shortly thereafter, Strong reported that live-attenuated vaccines protect humans from bubonic disease [62,63]. In subsequent years, live-attenuated vaccines were administered to tens of millions of humans in Indonesia, Madagascar and Vietnam [64]. Placebo-controlled clinical studies were not reported, but experimental studies, supported by field observations, strongly suggest these vaccines protect humans against both bubonic and pneumonic plague [54,58,64]. Unfortunately, these vaccines can be unstable and retain significant virulence in nonhuman primates, sometimes killing experimental animals [21,58,65,66]. In addition, they frequently cause debilitating fever, malaise and lymphadenopathy in humans [57]. These safety concerns have limited enthusiasm for the development of live-attenuated vaccines in the USA and Europe, where plague is uncommon and the risk of harm may outweigh the benefits of vaccination.

In the former Soviet Union, significant research effort was devoted to biodefense, as well as a biowarfare [50]. Live-attenuated plague vaccines were studied extensively in this regard [57, 67]. The currently favored vaccine is based on variants of pigmentation-negative *Y. pestis* strain EV 76. Despite safety concerns and a high degree of immune variability among vaccine recipients, the NIIEG line of EV 76 is still in use today [50].

Subunit vaccines based on the F1 & LcrV proteins

In 1952, Baker and colleagues purified the capsular F1 protein [68]. They demonstrated that rabbits immunized with purified F1 produce serum that agglutinates plague bacilli, and that vaccination with F1 protects mice and rats from subcutaneous challenge with virulent *Y. pestis* [68]. Ehrenkranz and Meyer subsequently demonstrated that vaccination with F1 protects macaques against pneumonic plague, as does passive transfer of serum collected from F1-vaccinated rabbits [69]. Subsequently, vaccination with recombinant F1 was shown to protect mice against aerosolized *Y. pestis* [70], as was passive transfer of an F1-specific monoclonal antibody [71]. Notwithstanding the importance of these landmark findings, it is now clear that virulent F1-negative *Y. pestis* strains exist [33-36,72], so vaccines based solely upon F1 will likely fail to protect against weaponized pneumonic plague.

Unlike F1, the multifunctional LcrV protein is critical for virulence [6,18,24,25,73-76]. A series of studies established that vaccination with purified LcrV protects mice against subcutaneous challenge, as does the passive transfer of LcrV-specific polyclonal and monoclonal antibodies [18,75-79]. After its cloning [80] and stable expression [79,81], vaccination with recombinant LcrV was shown to protect mice against aerosol infection with either F1-positive or F1-negative *Y. pestis* strains [82,83]. Moreover, a LcrV-specific monoclonal antibody passively protects mice against aerosolized *Y. pestis*, even when administered 48 h postinfection [84]. Notwithstanding these important achievements, vaccines based on LcrV alone may also fail to protect against weaponized pneumonic plague, because pathogenic *Yersinia* species express LcrV variants that may not confer cross-protective immunity [85].

Vaccines containing both F1 and LcrV will be more difficult to circumvent. They also provide greater protection than vaccines comprised of either subunit alone [86,87]. The UK defense department demonstrated that an alum formulation of the *Y. pestis* F1 and LcrV proteins protects mice against pulmonary *Y. pestis* challenge [88,89]. In parallel studies, the US Army Medical Research Institute of Infectious Diseases (USAMRIID) demonstrated that an alum formulation of an engineered F1-LcrV fusion protein (rF1V) protects mice against pulmonary

challenge with either F1-positive or -negative *Y. pestis* strains [83,90]. Both vaccines have entered human trials [91,92]. They appear to be safe, well tolerated and immunogenic.

It is not ethical to challenge humans with pneumonic plague, so plague vaccines will be licensed by the US FDA in accordance with the 'Animal Rule', which requires safety and immunogenicity data in humans along with robust efficacy data in animal models that mimic the human disease [93]. With regard to plague, mouse and nonhuman primate models have received the most attention thus far. At the FDA-sponsored Plague Vaccine Workshop held in 2004, USAMRIID presented data from a series of F1/LcrV vaccine studies in two nonhuman primate species. A full transcript is available on the FDA website [201]. With regard to the chosen primate models, USAMRIID researcher Louise Pitt stated, "In comparing the African green monkey and the cynomolgus macaque to date, based on clinical signs, the disease progression, and the pathology, as well as the susceptibility in terms of an LD50, they are very similar...and both are very similar to what is known about human disease" [201]. USAMRIID then demonstrated that F1/LcrV-based vaccines protect cynomolgus macaques against aerosolized *Y. pestis* (efficacy ranged from 80-100% in five trials). However, these vaccines fail to adequately protect African green monkeys (efficacy ranged from 0-75% in five trials). Differences in antibody titers, as measured by ELISA, do not explain the variable efficacy of F1/LcrV-based vaccines in primates [94,201]. These observations raise substantial uncertainty as to whether F1/LcrV-based vaccines will provide humans with the effective protection observed in cynomolgus macaques, or the insufficient protection observed in African green monkeys. When specifically asked, "Where humans are on this scale?", Pitt replied, "I would not like to comment" [201].

Recent advances in plague vaccine design

A number of approaches are underway to improve the efficacy of F1/LcrV-based vaccines [54]. Some researchers aim to genetically modify the antigens, for example, by mutating a potentially disruptive cysteine residue in rF1V [95] or by deleting the immunosuppressive region of LcrV [96]. Others are exploring the use of alternate adjuvants since, to date, the publicly disclosed F1/LcrV vaccine primate studies have all used an alhydrogel-based adjuvant. In murine models, intranasal delivery of rF1V using either protollin [97] or heat-labile enterotoxin [98] adjuvants protects against aerosol challenge, as does intranasal vaccination with biodegradable microspheres coencapsulating F1 and LcrV [99]. F1 vaccination using flagellin adjuvant also protects mice against intranasal challenge and induces robust F1-specific IgG responses in monkeys [100]. A DNA vaccine encoding LcrV also protects mice against intranasal *Y. pestis* challenge [101].

The efficacy of F1/LcrV-based vaccines may also be improved by delivering the antigens via live vectors suitable for human use. A single oral dose of attenuated salmonella expressing F1 and/or LcrV protects mice against subcutaneous [102-105] and intranasal challenge [106]. An intranasal prime-boost regimen with vesicular stomatitis virus expressing LcrV protects mice against intranasal challenge [107], and a single intramuscular vaccination with adenovirus expressing LcrV protects mice against intranasal challenge for at least 6 months [108].

These new approaches are certainly promising but, as already noted, F1-negative *Y. pestis* strains exist [33-36,72] and pathogenic *Yersinia* species express multiple LcrV variants, including some that may not confer cross-protective immunity [85]. Thus, bio-weapon engineers may circumvent vaccines based exclusively on F1 and LcrV. One solution to this concern is the incorporation of additional antigens into the rF1V vaccine. Vaccinating mice with *Yersinia* protein kinase A prolongs time to death after subcutaneous challenge with an F1-negative strain [109], and vaccinating with YopD increases survival in that model [109]. Vaccinating mice with a complex of YopBDE protects against intravenous challenge with an

F1-negative, pigmentation-negative strain [BLISKA J, PERSONAL COMMUNICATION]. Immunizing mice with *Yersinia* secretion protein F also protects against intravenous challenge with pigmentation-negative *Y. pestis* [110], as well as subcutaneous challenge with a fully virulent strain [111].

Plague vaccines based on live attenuated *Y. pestis* provide the theoretical advantage of simultaneously priming immunity against many antigens, thereby greatly reducing the likelihood of circumvention by weapon engineers. The original live attenuated strains were generated by selection, rather than precise genetic manipulation, thus raising concern about their genetic composition and stability. The widely used EV 76 strain can regain virulence and cause disease in primates [65]. Nevertheless, as recently as 2002, USAMRIID researchers noted, 'Despite their drawbacks, there is ample evidence that live-attenuated strains of *Y. pestis* should be considered as potential vaccine candidates' [21]. They suggested that combining multiple defined mutations should lead to safer live vaccines, and reported that a *Y. pestis* strain with defined mutations in both the pigmentation and plasminogen activator loci safely induces humoral responses in African green monkeys [21]. Unfortunately, USAMRIID has yet to report either mouse or primate protection data using this live-attenuated strain [21].

Several other groups have reported protection data on new live-attenuated vaccine strains with well-defined genetic modifications. Intranasal vaccination with a highly attenuated *Y. pestis* YopH mutant protects mice against both subcutaneous and pulmonary challenge [112], and a *pcm* mutant confers greater protection than EV 76 in a murine model of bubonic plague [113]. Montminy and colleagues have taken a different approach [114]. Following-up on prior reports that yersiniae express weakly inflammatory lipopolysaccharide when grown at 37°C [115,116], they engineered a *Y. pestis* strain that constitutively produces a more inflammatory lipopolysaccharide. A single subcutaneous vaccination with this highly attenuated strain protects mice against both subcutaneous and intranasal challenge [114]. These novel approaches should bolster enthusiasm for the development of safe and effective live-attenuated plague vaccines.

Antibody-mediated defense against plague

As noted above, the licensing of pneumonic plague vaccines will rely on efficacy data from animal studies and safety data from human trials. In addition, licensure will require assays that bridge the gaps between animal and human studies [93]. Specifically, we will need surrogate assays of vaccine efficacy so that clinicians can identify vaccination conditions (e.g., dose, frequency and route) that prime protective immune responses in humans. A detailed understanding of how vaccines protect against plague should inform the design of next-generation vaccines, while also identifying correlates of, and surrogate assays for, protection.

Antibodies clearly contribute to defense against plague but the mechanisms by which they do so *in vivo* remain to be established. It has long been recognized that serum from plague convalescents can passively transfer protection to naive mice, and an influential study by Green and colleagues demonstrated that passive transfer of F1/LcrV-specific antiserum provides immunodeficient mice with significant protection against aerosolized *Y. pestis* [117]. Given this documented efficacy of humoral immunity, even in animals lacking the capacity to mount acquired immune responses, pneumonic plague vaccine efforts have aimed, by and large, to stimulate high-titer, F1/LcrV-specific antibody responses. However, prechallenge F1/LcrV ELISA titers did not correlate with protective efficacy in the primate studies described previously [94]. Moreover, some vaccinated primates succumbed to challenge despite possessing high-titer antibodies [94]. These observations, along with many supporting studies in rodents, strongly suggest that antibodies titers alone, at least as measured by standard ELISA, will not suffice in predicting the efficacy of F1/LcrV vaccines in humans.

Since virulent F1-negative strains exist, most attention has focused on defining the mechanisms by which LcrV-specific antibodies confer protection. Antagonism of LcrV, either by genetic disruption or by specific antibody, converts the unimpeded growth of *Y. pestis* within tissues to a more confined, granulomatous response [6]. These impacts were originally ascribed to LcrV antibody-mediated suppression of LcrV-induced production of anti-inflammatory IL-10 [6,25]. However, recent reports question the importance of this pathway by demonstrating that *Y. pestis* LcrV is a weak inducer of IL-10 [39,40] and that LcrV-specific antibody retains its capacity to limit bacterial growth in IL-10-deficient mice [118]. Nevertheless, LcrV antagonism does seem to permit the resolution of *Y. pestis* infections through a process associated with granuloma formation.

Another, nonmutually exclusive, mechanism by which LcrV-specific antibodies may confer protection is by countering the LcrV-mediated suppression of neutrophil chemotaxis [38]. This possibility is supported by a recent report demonstrating that LcrV-specific antibody loses its capacity to limit bacterial growth in neutrophil-depleted mice [119].

LcrV-specific antibodies also help macrophages [119,120] and neutrophils [119] phagocytose *Y. pestis* bacilli *in vitro*. This activity likely involves an opsonophagocytic mechanism since LcrV is expressed on the bacterial surface [121,122], and early opsonophagocytic studies by Jawetz and Meyer concluded that immune serum enables phagocytes to kill *Y. pestis* bacilli [26,27]. DynPort Vaccine Company LLC intends to use an opsonophagocytic assay to bridge animal and human studies as they manage the clinical development of the rF1V vaccine [92]. However, DynPort has yet to demonstrate that this assay provides a reliable correlate of protection.

Multiple groups have recently investigated whether the ability of LcrV-specific antibodies to neutralize yersiniae-induced cytotoxicity might serve as a correlate of immunity to *Y. pestis* infection [94,123]. Contact with *Y. pestis* can activate macrophage apoptosis *in vitro* through a Yop-dependent mechanism [120]. LcrV-specific antibodies suppress both Yop-translocation [118,119,121] and macrophage apoptosis [94,120]. In combination with the opsonophagocytic mechanisms described above, these findings suggest that LcrV-specific antibodies promote phagocytosis in a manner that enables phagocytes to ingest *Y. pestis* bacilli, without themselves being killed in the process. USAMRIID researchers developed a flow cytometry-based cytotoxicity assay using the human HL60 cell line and a modified strain of *Y. pseudotuberculosis* that expresses the *Y. pestis* LcrV protein [94]. LcrV-specific antibodies suppress cell death in this assay and the capacity of sera from rF1V-vaccinated mice to suppress cell death is a significant predictor of survival after subcutaneous challenge with F1-negative *Y. pestis*. Likewise, there is an association between suppression of cell death and survival when analyzing sera from rF1V-vaccinated non-human primates that had been aerosol challenged. It must be noted, however, that sera from many of the surviving primates apparently failed to suppress cell death [94]. These false negatives raise some concern but this study provides a promising foundation for the development of surrogate assays for antibody-mediated protection against plague.

Cell-mediated defense against plague

By comparison with acquired humoral immunity, roles for acquired cellular immunity during vaccine-mediated defense against plague have received relatively little attention. Meyer and colleagues found that immune serum suffices to enhance phagocytosis but “cooperation of immune serum and immune cells is necessary for the efficient destruction of bacteria” [26]. In 1977, Wong and Elberg replicated and extended these observations [124]. They cultured spleen cells from immune mice with killed *Y. pestis*, harvested supernatant, and then exposed naive phagocytes to the supernatant. They found that immune spleen cells produce soluble factors

that protect phagocytes from cytolysis upon subsequent exposure to viable *Y. pestis*. Depletion studies suggested that splenic T cells play a key role in generating this protective activity.

Our current understanding of T-cell-mediated cellular immunity readily accommodates these historical studies. Cytokine products of T cells, most notably IFN- γ and TNF- α , are known to activate phagocyte antimicrobial activities, including production of reactive oxygen and reactive nitrogen. *Y. pestis* replicates within naive macrophages [30,42,74] and in macrophages exposed to IFN- γ after infection [125]. However, pretreatment of macrophages with IFN- γ and TNF- α restricts intracellular replication [45]. In combination with the aforementioned studies of antibody-mediated protection, these *in vitro* findings suggest that antibodies help phagocytes internalize *Y. pestis*, while cytokine products of T cells enable phagocytes to survive *Y. pestis* encounters and kill internalized bacilli (Figure 1).

In vivo studies also support the notion that cytokines play important roles during humoral defense against plague. Vaccination stimulates robust production of F1/LcrV-specific antibodies in STAT4-deficient mice, yet these animals, which are impaired for production of IFN- γ , are poorly protected against subcutaneous *Y. pestis* challenge [126]. Likewise, we recently demonstrated that optimal antibody-mediated protection against pulmonary challenge with pigmentation-negative *Y. pestis* requires host production of IFN- γ , TNF- α and inducible nitric oxide synthase [127].

As noted above, vaccination with LcrV appears to protect against plague, at least in part, by facilitating granuloma formation, a hallmark of cellular immunity [6]. Infection with mutant *Y. pestis* strains lacking expression of LcrV and/or certain Yops also induces granuloma formation [6,128,129]. Altogether, these findings suggest that neutralization of *Y. pestis* virulence factors enables classical cellular defense mechanisms to effectively combat plague.

Y. pestis actively combats cellular immunity via a number of mechanisms. Recent studies reveal that *Y. pestis* produces factors that antagonize host production and use of reactive nitrogen [125,130]. In addition, the Yops may suppress T-cell responses, both by directly targeting T cells and by targeting dendritic cells [6,24,25,37]. This deliberate impairment of acquired cellular immunity, coupled with the exceptionally rapid course of plague, suggests that T cells might not have an opportunity to contribute to protection, particularly in the absence of neutralizing antibodies. Nevertheless, Alonso and colleagues demonstrated that passive transfer of cells, but not sera, from *Y. enterocolitica* convalescent mice partially protects naive mice against subcutaneous *Y. pestis* challenge [131]. Although the protective cell was not identified, these studies suggested protection by cross-reactive T cells. Likewise, Wake and Sutoh demonstrated that T cells contribute to vaccination, although they did not dissociate the cellular functions of T cells from their capacity to help B cells produce antibody [132]. Recently, we demonstrated that vaccination with live *Y. pestis* primes T cells that passively transfer protection [20,133]. Notably, neither F1 nor LcrV appear to be dominant antigens recognized by T cells primed by live *Y. pestis* infection [133].

In some settings, cellular immunity may even suffice to protect against plague. It is certainly appreciated that vaccinated animals often survive challenge despite low-antibody titers. For example, live-attenuated *Y. pestis* vaccines solidly protect guinea pigs against plague, without eliciting significant antibody titers [64], and monkeys immunized with the live-attenuated EV 76 vaccine 'not infrequently survived challenge...with little antibody measurable' [69]. Moreover, Nakajima and Brubaker demonstrated that injecting naive mice with IFN- γ and TNF- α suffices to protect against intravenous challenge [78] and we demonstrated that antibody-deficient μ MT mice can be vaccinated effectively against pneumonic challenge [20]. Both our studies and those of Nakajima and Brubaker employed pigmentation-negative *Y. pestis* as challenge, which is notable because Pujol and colleagues recently demonstrated

that *ripA*, a gene within the pigmentation locus, suppresses the production of antimicrobial nitric oxide by IFN- γ -activated macrophages [125]. Thus, pigmentation-deficient *Y. pestis* may be more sensitive to cell-mediated defense mechanisms than are pigmentation-positive *Y. pestis*. Nonetheless, Elvin and Williamson's studies of vaccinated STAT4-deficient mice certainly suggest that defense against fully virulent *Y. pestis* also requires host production of type 1 cytokines, such as IFN- γ and TNF- α [126].

Expert commentary

Y. pestis has evolved to powerfully resist phagocytes, thereby overcoming host defense. LcrV is a key mediator of this phagocytic resistance; it blocks neutrophil chemotaxis, facilitates the translocation of phagocyte-debilitating Yops, suppresses granuloma formation and may exert other anti-inflammatory activities as well. In some experimental models, LcrV-specific antibodies suffice to protect against pneumonic plague, apparently by promoting phagocytosis and helping phagocytes survive *Y. pestis* encounters. In other models, high-titer LcrV-specific antibodies do not seem to suffice. In those contexts, phagocytes may require additional help from activated T cells. Specifically, T cells may need to produce cytokines, such as IFN- γ and TNF- α that activate phagocytes, restrict intracellular *Y. pestis* replication and facilitate the killing of intracellular bacilli. Figure 1 presents a model depicting how IFN- γ and TNF- α may augment antibody-mediated defense. An increasing number of studies support this model by demonstrating the roles of IFN- γ , TNF- α and nitric oxide during defense against plague [20, 44,45,78,125,127,130]. This is one reason I advocate that pneumonic plague vaccines should strive to prime both humoral and cellular immunity. I also suggest that pneumonic plague vaccine trials should now aim to assay T cells and their cytokines, as well as antibodies.

The licensure of a safe and effective pneumonic plague vaccine will need to overcome a number of hurdles. Most importantly, it will need to meet the safety and efficacy requirements set forth by the FDA's 'Animal Rule'. In my opinion, the Animal Rule provides a reasonable path to plague vaccine licensure but encourages studies of the 'easy' animal models, where candidate vaccines work well, while inadvertently discouraging research on the difficult models, where candidate vaccines fail. This path may produce a safe and licensable vaccine, but will it really protect humans? More specifically, how can the FDA ensure that the rF1V vaccine will provide humans with the effective protection observed in cynomolgus macaques, rather than the insufficient protection observed in African green monkeys? Given that efficacy studies cannot be performed in humans, this question can only be addressed through experimental research. As stated by Meyer in 1974, 'It is an exciting challenge to explore the place of man in the spectrum of susceptibility to plague... We must remember, however, that an infallible means of evaluating hereditary factors that influence resistance or susceptibility to infection is yet to be developed' [65]. At present, we remain unable to predict exactly where the diverse human population resides on the evolutionary scale of plague susceptibility. As such, I believe that we need a vaccine that works in multiple primate species or, at least, we need to understand why the vaccine works in some primates, but not others.

An unfortunate consequence of biotechnology is that weapons' engineers can potentially circumvent years of vaccine research and development. Thus, the rF1V vaccine, even if reformulated, readjuvanted and/or supplemented with other antigens, may fail to protect against cleverly weaponized *Y. pestis* strains. On the other hand, the live-attenuated EV 76 vaccine may provide a valuable near-term solution to pneumonic plague biowarfare threats. Concerns about safety, not the efficacy, have curbed the wide-scale use of live-attenuated *Y. pestis* vaccines [54,65]. Nevertheless, live *Y. pestis* vaccines have been administered to tens of millions of humans, apparently without causing deaths [64] and the EV 76 vaccine is still in use today in the former Soviet Union [50]. Undoubtedly, EV 76 can produce serious and unpleasant reactions, and EV 76-like vaccines have killed African green monkeys [21,65].

However, I find it ironic that we are uncomfortable with the procurement of EV 76-like vaccines because they have harmed African green monkeys, yet we are comfortable advancing the rF1V vaccine, which fails to protect African green monkeys. If the argument for ignoring the failure of the rF1V vaccine in African green monkeys is that such animals are not valid models of plague in humans, then certainly we should consider them questionable models for virulence testing. Thus, I advocate that we stockpile EV 76 for emergency use and, in parallel, devote significant effort to the development of less reactogenic, more stable, genetically defined, live-attenuated vaccines.

Similar to EV 76, the pigmentation-negative KIM5 strain used in our vaccine studies expresses LcrV and Yops. These factors can certainly suppress phagocytic responses, and may have the capacity to suppress T-cell responses [6,24,25]. Nevertheless, our studies indicate that vaccination with KIM5 primes *Y. pestis*-specific memory T cells [133]. Thus, suppression of T-cell responses by the LcrV and Yops is, at most, incomplete. In suitably vaccinated animals, antibodies specific for LcrV, and perhaps other *Y. pestis* proteins as well, should slow disease progression and allow time for the activation, expansion and recruitment of memory T cells. In turn, these T cells should activate and amplify phagocyte defense mechanisms, thereby degrading intracellular niches for *Y. pestis* survival, while encouraging the formation of protective granulomas. Given such potentially synergistic interactions, and given that humoral and cellular immunity often deploy complementary defense mechanisms, I believe that next-generation plague vaccines, whether live-attenuated or subunit-based, should strive to prime both humoral and cellular immunity.

Five-year view

The safety of F1/LcrV-based vaccines in humans should soon be established since clinical trials are now underway. Further research should lead to the development of robust correlate assays for vaccine efficacy in mice and nonhuman primates. These assays will then serve as surrogates for protection studies in humans. In that capacity, correlate assays will enable human clinical trials to determine vaccine formulations, dosages and schedules that best prime protective responses.

Additional plague vaccine antigens should be identified and validated. Incorporating additional antigens into F1/LcrV-based vaccines will reduce opportunities for circumvention by weapon engineers.

A better understanding of virulence mechanisms that operate within the lung during infection should reveal new targets for pneumonic plague vaccines and therapeutics. Prior research on *Y. pestis* virulence has focused primarily on bubonic and septicemic models. Septicemia is certainly relevant to pneumonic plague since it accompanies both bubonic and pneumonic infections. However, given present biowarfare concerns, which envision deployment of aerosolized *Y. pestis*, there is now a greater need to explicitly characterize virulence factors that impact pulmonary disease.

It is my hope that research on the development of new, improved live-attenuated vaccines will continue and will be strongly encouraged by funding agencies. Ideally, these vaccines must be stable, fully defined at the molecular level and sufficiently attenuated to minimize vaccine sequelae, both in animals and in humans.

Key issues

- We currently lack a safe, effective and licensed vaccine for pneumonic plague, which is nearly always fatal and has been weaponized.

- *Yersinia pestis* overcomes host defense, in a large part, by debilitating phagocytes.
- The leading candidate subunit vaccines - combinations of the *Y. pestis* F1 and LcrV proteins - generate antibodies that help phagocytes combat *Y. pestis*. However, these vaccines have failed, thus far, to adequately protect all primate species. Even with improvement, F1/LcrV-based vaccines may fail to protect against weaponized strains of *Y. pestis*.
- Live-attenuated *Y. pestis* vaccines protect against pneumonic plague, probably by generating both protective antibodies and protective T cells. These live vaccines raise significant safety concerns but should be effective against weaponized strains of *Y. pestis*.
- Further research should produce improved vaccines by further defining precisely how vaccine-primed immunity combats pneumonic plague and by identifying *Y. pestis* virulence mechanisms that operate within the lung. These studies should also help to define robust correlates of vaccine efficacy that can serve as surrogates for efficacy in humans.

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Figure 1. Model depicting how cellular immunity may augment antibody-mediated defense against pneumonic plague

(A) In naive mammals, *Yersinia pestis* bacilli (small solid spheres) grow unimpeded. Virulence factors target and debilitate PMN phagocytes and Mac. The Macs are permissive for intracellular growth of *Y. pestis*. (B) In the presence of antibodies that target virulence factors, opsonized bacilli are phagocytosed and destroyed (small striped spheres) by PMN. Mac phagocytose bacteria as well, but remain permissive for intracellular replication. (C) In the presence of antibodies and cellular immunity, T cells produce cytokines (IFN- γ , TNF- α) that activate Macs, causing them to upregulate production of iNOS and other antibacterial mechanisms, thus, killing bacilli and degrading intracellular niches for *Y. pestis* replication. iNOS: Inducible nitric oxide synthase; Mac: Macrophage; PMN: Polymorphonuclear.