Hereditary Hemochromatosis Restores the Virulence of Plague Vaccine Strains

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Nonpigmented Yersinia pestis (pgm) strains are defective in scavenging host iron and have been used in live-attenuated vaccines to combat plague epidemics. Recently, a Y. pestis pgm strain was isolated from a researcher with hereditary hemochromatosis who died from laboratory-acquired plague. We used hemojuvelin-knockout (Hjv−/−) mice to examine whether iron-storage disease restores the virulence defects of nonpigmented Y. pestis. Unlike wild-type mice, Hjv−/− mice developed lethal plague when challenged with Y. pestis pgm strains. Immunization of Hjv−/− mice with a subunit vaccine that blocks Y. pestis type III secretion generated protection against plague. Thus, individuals with hereditary hemochromatosis may be protected with subunit vaccines but should not be exposed to live-attenuated plague vaccines.

Yersinia pestis is the causative agent of plague, an ancient scourge that precipitates intermittent pandemics [1, 2]. Plague is a disease of mammals that is transmitted by flea bite or aerosol droplets, causing either bubonic or pneumonic plague [3]. Inoculation of humans with live-attenuated Y. pestis strains, identified as nonpigmented (pgm) colonies on hemin agar, affords protection against bubonic and pneumonic plague [4]. Nonpigmented strains, most notably Y. pestis EV76, have been used extensively as live-attenuated vaccines to successfully stem the spread of the last plague pandemic in Asia [5].

The pathogenesis of Y. pestis infections relies on the pathogen’s type III secretion pathway, which delivers Yop effectors into immune cells via type III needle complexes that are capped by the protective antigen LcrV [6–8]. The protective immunity raised by live-attenuated plague vaccines is based on antibodies that are directed against the fraction 1 capsular antigen (Caf1, or F1 pilus) of Y. pestis and that interfere with the delivery of effectors by LcrV-capped type III needle complexes [9, 10]. Y. pestis variants defective for the expression of F1 capsular antigen caused plague in animals that had been immunized with the live-attenuated vaccine, indicating that protection is not universal for all Y. pestis isolates [10].

Spontaneous excision and loss of the 102-kb high-pathogenicity island and pigmentation (pgm) locus occurs on the Y. pestis chromosome at flanking IS100 elements [11, 12]. Y. pestis pgm strains are attenuated in animal models of bubonic and pneumonic plague because of their inability to synthesize yersiniabactin, a siderophore that scavenges iron from transferrin during host infection [13, 14]. Live-attenuated pgm plague vaccine–related illnesses were not reported during the immunization campaign involving Asian populations [5]. However, in a small clinical trial in the United States, some recipients of the live-attenuated plague vaccine experienced malaise, fever, and severe reactogenicity requiring hospitalization [4]. Recently, the nonpigmented Y. pestis strain UC91309 was isolated from a researcher with fatal, septicemic plague [15]. Genome sequencing suggested that the isolated strain had been acquired in the laboratory [15]. Autopsy findings included abnormally high levels of iron deposits in the liver and markedly elevated levels of serum ferritin, iron, total iron-binding capacity, and iron saturation [15]. The presence of a HFE
C282Y mutation supported the postmortem diagnosis of hereditary hemochromatosis [15]. These clinical and pathological findings suggested that iron overload in tissues of an individual with hereditary hemochromatosis may complement the iron-scavenging defect in mutants that are unable to synthesize yersiniabactin, thereby restoring the virulence of pgm variants and their ability to cause plague.

Formal proof for this hypothesis is not yet available. To address this question, we analyzed the virulence properties of Y. pestis UC91309 and used a mouse model of hereditary hemochromatosis to examine whether increased iron load in host tissues may restore the virulence of nonpigmented Y. pestis strains. Further, we asked whether the rV10-2 plague subunit vaccine [16], which elicits LcrV-specific antibodies that block Y. pestis type III injection of immune cells [17], can protect mice with hereditary hemochromatosis against plague disease.

METHODS

Expression of LcrV and F1 by Plague Strains
Y. pestis strains UC91309 [15], KIM D27 [18], CO92 [17], KIM D27 ΔlcrV, and KIM D27 Δcafl [9] were grown in brain-heart infusion broth. Cultures were centrifuged, and proteins in the supernatant and bacterial pellet were precipitated with trichloroacetic acid (10%), washed in ice-cold acetone, dried, and suspended in sample buffer. Proteins were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, electro-transferred to PVDF membrane, and immunoblotted with rabbit antibodies specific for Caf1, LcrV, or RpoA [9].

Colony Pigmentation
Y. pestis strains were streaked onto blood agar plates containing Congo red. Wild-type strains, such as the fully virulent Y. pestis isolate CO92, form bright red colonies on blood agar with Congo red, whereas pgm strains (KIM D27 and UC91309) appear pale pink.

Histopathologic Analysis
Animal tissues obtained during necropsy were fixed in 10% neutral buffered formalin and embedded. Blocks were sectioned (5-µm sections) and stained with hematoxylin-eosin or Prussian blue prior to microscopy and image analysis.

Hemjuvelin-Knockout (Hjv<sup>−/−</sup>) Mice
Hjv<sup>−/−</sup> mice (129S6/SvEvTac) were bred at the University of Chicago; 6–8-week-old females were transferred to the Howard Taylor Ricketts Laboratory. Cohorts of 129S1/SvImJ wild-type (Hjv<sup>+/+</sup>) mice (stock 002448) were purchased from Jackson Laboratory at 6–8 weeks of age. All animals were fed Teklad Global 18% protein rodent diet (Harlan Laboratories) with standard iron content (200 mg iron/kg feed) throughout the experiments. Five-millimeter pieces of frozen mouse tail were used for genotyping by polymerase chain reaction with the following primers: Hjv<sup>−/−</sup> forward, 5′-CGG ATG GTT TTT GCC AGT TAG-3′; Hjv<sup>−/−</sup> reverse, 5′-GCC TTT ACG ATA TCT CAG TCC-3′; wild-type forward, 5′-GAA TGG CTT CCT TCC ATC AA-3′; and wild-type reverse: 5′-ATC TTC AAA GGC TGC AGG AA-3′. For iron analysis, blood was collected via cardiac puncture from 8-week-old mice and captured in serum separator tubes (Becton Dickinson), and iron concentration was determined with the iron/UIBC kit (Roche Diagnostics).

Plague Subunit Vaccine
Mice (wild-type or Hjv<sup>−/−</sup>) were immunized with clinical grade rV10-2 vaccine (100 µL phosphate-buffered saline [PBS] with 25 µg rV10-2 and 250 µg Alhydrogel) on day 0 and day 21 via intramuscular injections [16]. By using dose escalation studies, earlier work demonstrated that 25 µg rV10-2 adsorbed to 250 µg aluminum hydroxide elicits antibody responses and protective immunity in mice [16]. As controls, animals received mock vaccinations (100 µL PBS with 250 µg Alhydrogel). Mice (n = 5) were bled, and levels of antigen-specific serum immunoglobulin G (IgG) were determined on day 0 (prior to the first immunization) and day 42. rV10-2-specific IgG was quantified by enzyme-linked immunosorbant assay in microtiter plates precoated with peroxidase AffiniPure IgG (H + L) (Jackson ImmunoResearch Laboratories) [19]. Statistical analysis of antibody levels was performed in pair-wise comparison using the unpaired 2-tailed Student t test. Forty-two days after immunization, animals (n = 10) were challenged with Y. pestis CO92 or Y. pestis KIM D27 via subcutaneous injection.

Experimental Plague Challenge
Swiss Webster mice (n = 10; 6–8 week-old females; Charles River Laboratory) were infected with Y. pestis isolates. To prepare the challenge dose for the bubonic plague model, 3 mL of heart infusion broth was inoculated with colony material of plague strains and grown at 26°C overnight. Culture density was calculated via absorbance of 600 nm light (A<sub>600</sub> 1 is equivalent to 2.17 × 10<sup>8</sup> plague bacteria). Cultures were diluted in sterile PBS to the desired dosage, and 100 µL of a bacterial suspension was injected subcutaneously. Aliquots of inoculum were spread on plates containing Congo red and heart infusion broth to enumerate challenge doses. Infected animals were monitored for survival over 14 days. Animals that died from infection and survivors were subjected to necropsy. Organ tissues were either homogenized and spread on agar plates for colony formation and enumeration or subjected to histopathologic analysis. Animals that were pretreated with iron received an intraperitoneal injection of 0.1 mL 50% (v/v)
iron dextran (100 mg/mL) and 50 mg/mL desferrioxamine B mesylate.

For the pneumonic plague model, animals were anesthetized by intraperitoneal injection with a cocktail of ketamine (Ketaved; Vedco) and xylazine (Sigma) and challenged by intranasal inoculation with 20 µL of Y. pestis suspension. For these experiments, Y. pestis was grown in heart infusion broth supplemented with 2.5 mM of calcium at 37°C overnight, conditions that mimic the ambient temperature during man-to-man transmission of pneumonic plague. Plague bacilli were washed and diluted in sterile PBS to the required concentration. All infected animals were observed over 14 days for morbidity, mortality, or recovery. Analysis of the statistical significance of mortality studies was performed using the 2-tailed log-rank (Fisher exact test) and GraphPad Prism4 software.

**Vertebrate Animals and Select Agent Research**

Animal experiments were performed in accordance with institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee, Select Agent Committee, and the Institutional Animal Care and Use Committee. Experiments with Y. pestis CO92 (risk group 3 pathogen) and with KIM D27 and UC91309 (risk group 2 pathogens) were performed in BSL-3 and ABSL-3 at the Howard Taylor Ricketts Laboratory.

**RESULTS**

**Virulence Attributes of Y. pestis Strain UC91309**

Genome sequencing revealed a close relationship between Y. pestis KIM D27 and UC91309, the absence of the 102-kb high-pathogenicity island/pgm locus, and the presence of 3 virulence plasmids that encode for the type III secretion pathway (pCD1), the F1 pilus operon (pMT1), and Pla protease (pPPC1) [15]. In contrast to wild-type strain Y. pestis CO92, UC91309 and KIM D27 formed nonpigmented colonies on Congo red agar (Figure 1A). Immunoblot analysis of extracts derived from UC91309 generated positive signals for the expression and secretion of LcrV, the protective antigen that is secreted via the type III pathway, and for F1 pili (Figure 1B). In contrast to Y. pestis CO92, KIM D27 and UC91309 were unable to cause lethal plague infection in Swiss Webster mice at a subcutaneous challenge dose of 10^5 colony-forming units (CFUs) (Figure 1C). At a higher challenge dose (>10^6 CFUs), we observed some mortality in infected mice, demonstrating that the 2 strains were attenuated but did not display a difference in residual virulence (P = .6416; Figure 1D).

Earlier studies revealed that animals pretreated with iron salts developed plague when challenged with nonpigmented Y. pestis isolates [20, 21]. Injection of Swiss Webster mice with 10 mg/mL iron dextran into the peritoneal cavity restored some, but not all, of the virulence of Y. pestis KIM D27 and UC91309 (Figure 1D). We did not observe a significant
difference between KIM D27 and UC91309 virulence in iron dextran–treated animals ($P = .31$; Figure 1D). Histopathologic features of animals that succumbed to challenge included scattered hepatic granulomas with infiltrates of polymorphonuclear neutrophils and histiocytes (Figure 2). Histiocytes harbored intracellular hemosiderin deposits, a consequence of iron dextran administration. Spleen tissues displayed extensive coagulative necrosis with fibrin and neutrophil debris (Figure 2). Of note, iron dextran accumulates in the reticuloendothelial system and impairs phagocyte function [22]; mice that receive iron dextran therefore cannot be used as a model of iron overload in tissue, which occurs in individuals with hereditary hemochromatosis.

Plague Disease in Mice With Hereditary Hemochromatosis

$Hjv^{-/-}$ mice were used as a model for hereditary hemochromatosis [23]. $Hjv^{-/-}$ animals accumulate iron in liver, pancreas, and heart tissues at levels resembling those associated with hereditary hemochromatosis in humans [23] (Figure 3A). As expected, $Hjv^{+/+}$ mice displayed an increased concentration of iron in serum (Figure 3B). Prussian blue staining of fixed, thin-sectioned organ tissues revealed iron deposits in the liver but not in the spleen of $Hjv^{-/-}$ mice (Figure 3A) [23]. As a control, liver tissues from wild-type mice did not contain iron deposits (Figure 3A). Subcutaneous challenge of $Hjv^{-/-}$ mice with 4 or 20 CFUs of wild-type $Y. pestis$ CO92 caused lethal bubonic plague infections in 30% and 70% of challenged animals, respectively (Figure 3C). Subcutaneous challenge of wild-type mice with 4 or 20 CFUs generated similar results (wild-type vs $Hjv^{-/-}$ mice, $P = .34$). Intranasal instillation of 340 or 10 000 CFUs of $Y. pestis$ CO92 into wild-type mice caused lethal pneumonic plague in 20% and 100% of all challenged animals, respectively. These results are similar to measurements for pneumonic plague in BALB/c mice inoculated intranasally with $Y. pestis$ CO92; the median lethal dose for pneumonic plague in BALB/c mice is 389 CFUs [17]. Similarly, instillation of $Hjv^{-/-}$ mice with 340 or 10 000 CFUs of $Y. pestis$ CO92 caused lethal pneumonic plague in 40% and 100% of infected animals, respectively (wild-type vs $Hjv^{-/-}$ mice, $P = .24$; Figure 3D). Thus, the virulence of wild-type $Y. pestis$ CO92 was not altered in $Hjv^{-/-}$ mice with hereditary hemochromatosis.
Virulence of Nonpigmented Plague Strains in \( Hjv^{-/-} \) Mice

Wild-type and \( Hjv^{-/-} \) mice were challenged by subcutaneous injection with \( 10^5 \) or \( 10^7 \) CFUs of the nonpigmented plague strain \( Y. pestis \) KIM D27 (Figure 4). Wild-type mice displayed clinical signs of infection, but most animals recovered and survived (Figure 4A). In contrast, subcutaneous injection of \( Hjv^{-/-} \) mice with \( 10^5 \) or \( 10^7 \) CFUs of \( Y. pestis \) KIM D27 caused lethal plague disease in 70% of challenged animals on average (wild-type vs \( Hjv^{-/-} \) mice, \( P < .0001 \); Figure 4A). Similar results were obtained when \( Y. pestis \) UC91309 was used as a challenge strain (wild-type vs \( Hjv^{-/-} \) mice, \( P = .0001 \); Figure 4B). To monitor bacterial replication in infected tissues, animals were challenged with \( 10^6 \) CFUs of \( Y. pestis \) KIM D27 and euthanized at timed intervals. During necropsy, the local lymph node, spleen, and liver were removed, and bacteria in organ tissue homogenates were quantified by enumerating colonies on agar plates (Figure 4C). Subcutaneous injection of \( 10^6 \) CFUs of \( Y. pestis \) KIM D27 into wild-type mice was followed by dissemination of plague bacteria to the local lymph node within 24 hours. Dissemination of nonpigmented plague bacteria to the spleen and liver occurred infrequently, and bacteria were cleared from all host tissues within 7 days (Figure 4C). Subcutaneous injection of \( 10^6 \) CFUs of \( Y. pestis \) KIM D27 into \( Hjv^{-/-} \) mice caused early dissemination to the local lymph node, spleen, and liver within 24 hours (Figure 4D). Dissemination and replication of nonpigmented
plague bacteria in \( Hjv^{−/−} \) mice progressed steadily. Four days after challenge, plague bacteria were found in all organ tissues, and most animals succumbed to infection (Figure 4D).

Analysis of spleen and liver tissues from wild-type mice infected with \( Y. pestis \) KIM D27 revealed no signs of inflammation or infection (Figure 5). \( Hjv^{−/−} \) mice developed focal infiltrates of neutrophils and histiocytes in the liver (Figure 5). Of note, histiocytes did not harbor hemosiderin pigment, as was observed for wild-type animals treated with iron dextran. Spleen tissues of \( Hjv^{−/−} \) mice infected with the nonpigmented plague strain harbored focal inflammatory infiltrates composed of neutrophils and histiocytes (Figure 5).

**Subunit Vaccines Protect Mice With Hereditary Hemochromatosis Against Plague**

LcrV is a secreted pentameric protein that caps the needles of \( Y. pestis \) type III secretion machines [8]. LcrV is a protective antigen [24], and \( Y. pestis \) mutants with a deletion of lcrV are avirulent [25]. Immunization of animals with purified LcrV raises specific antibodies that block type III secretion and generate protection against plague [17, 26]. Because \( Y. pestis \) KIM D27 and UC91309 were endowed with functional type III secretion machines (Figure 1B), we wondered whether LcrV-based vaccines can protect \( Hjv^{−/−} \) mice against plague. rV10-2 vaccine lacks the immunomodulatory attributes of LcrV [19, 27] and protects mice, rats, guinea pigs, and nonhuman primates against plague [16]. Mice were immunized with either adjuvant alone (PBS) or with rV10-2 adsorbed to aluminum hydroxide [16]. Vaccinated animals produced rV10-2–specific IgG, whereas adjuvant control mice did not (mock vs rV10-2, \( P < .1 \times 10^{-5}; \) Figure 6A). Animals were challenged by subcutaneous injection with either 500 CFUs of \( Y. pestis \) CO92 (>50 median lethal doses) or 5 \times 10^6 CFUs of \( Y. pestis \) KIM D27. Mock-immunized \( Hjv^{−/−} \) mice succumbed to challenge with fully virulent \( Y. pestis \) CO92 (Figure 6B). In contrast, 90% of the hemochromatosis mice that received rV10-2 vaccine were protected from lethal plague challenge (mock vs rV10-2, \( P < .0001; \) Figure 6B). One \( Hjv^{−/−} \) animal died during this experiment with disease features that were not consistent with plague. rV10-2 immunization also protected \( Hjv^{−/−} \) mice against challenge with the nonpigmented plague strain, while 60% of the mock-immunized animals died from plague (mock vs rV10-2, \( P < .01; \) Figure 6B). Thus, rV10-2 vaccine protected \( Hjv^{−/−} \) mice with hereditary hemochromatosis against lethal plague caused by either wild-type or \( pgm \) mutant \( Y. pestis \).

**DISCUSSION**

Hereditary hemochromatosis results from mutations in genes that regulate iron uptake [28]. Five genes have been associated with disease, including \( HFE \) [29], \( TRF2 \) (transferrin receptor-2) [30], \( SLC40A1 \) (ferroportin) [31], \( HAMP \) (hepcidin) [32], and \( HVJ \) (hemojuvelin) [33]. Hepcidin is a small, secreted peptide that functions as a hormone [34]. Its abundance is elevated when hepatocytes respond to increased iron stores [35]. Hepcidin associates with ferroportin, a membrane protein of enterocytes and macrophages [36]. Macrophages scavenge iron
from senescent erythrocytes, whereas enterocytes retrieve iron from the intestine [28]. In both cell types, ferroportin is the membrane exporter for iron across the plasma membrane into body fluids [37]. Reduced abundance of hepcidin is correlated with disease severity due to increased iron in blood and in liver, heart, and pancreas tissues [28]. Hjv−/− mice recapitulate the clinical features of hereditary hemochromatosis in patients homozygous for autosomal recessive mutations in Hjv or Hamp [23]. Hemojuvelin functions as a coreceptor for bone morphogenetic protein in a signaling cascade with SMAD factors that control hepcidin expression [38]. The most frequent cause of human hemochromatosis is the autosomal recessive Cys282Tyr mutation in the HFE gene [29]. HFE associates with transferrin receptor to modulate the cellular uptake of iron from serum transferrin [39]. Patients homozygous for HFE C282Y, which occurs in 0.1% of white individuals but not in other ethnic groups, develop late-onset hemochromatosis with variable penetrance [40, 41]; environmental factors (eg, diet) and other genetic polymorphisms contribute to disease pathogenesis [42, 43]. Mice homozygous for HFE C282Y accumulate only small amounts of iron and are not a suitable model for hereditary hemochromatosis [44].

We show here that Y. pestis pgm strains, which have been used in live-attenuated plague vaccines, had some of their virulence-associated defects restored during infection of mice with hereditary hemochromatosis. Clinical trial data for live-attenuated plague vaccines are currently not available. Whether vaccine-related side effects or even death occur more frequently among white individuals than in other ethnic populations is not known [4]. Such a scenario would be expected if individuals with hereditary hemochromatosis were susceptible to plague infection with Y. pestis pgm strains. A recent report detailed the case of a researcher with hereditary hemochromatosis who had a fatal, septicemic plague infection with the nonpigmented strain Y. pestis UC91309 [15]. Autopsy revealed increased iron deposition (14,672 µg/g dry weight; hepatic iron index, 4.4 µmol/g per year) in noncirrhotic liver parenchyma [15] and increased serum iron levels (ferritin level, 392,530 ng/mL; total iron-binding capacity, 648 µg/dL; total iron, 541 µg/dL; and iron saturation, 83.5% [15]. Our results suggest that hereditary

Figure 5. Histopathologic analysis of mice with hemochromatosis that were infected with nonpigmented Yersinia pestis. Wild-type (WT) mice or hemojuvelin-knockout (Hjv−/−) mice (n = 5) were infected by subcutaneous injection with 1 × 10^6 colony-forming units (CFUs) of Yersinia pestis KIM D27 (pgm). On day 4 (for Hjv−/− mice) or day 7 (for wild-type mice), animals were euthanized, and necropsy was performed. Liver and spleen were removed, and the organs were fixed, embedded, thin sectioned, and stained with hematoxylin-eosin and analyzed by light microscopy with a 40 × objective. WT animals displayed the physiological architecture of spleen tissues with lymphoid follicles (LF) and sinusoids (SI; A) or liver tissues with healthy liver parenchyma (LP) and blood vessels (BV; C). Hjv−/− mice harbored focal inflammatory infiltrates composed of histocytes (black arrows) and neutrophils (blue arrows) in both liver and spleen tissues.
hemojuvelin-knockout (Hjv−/−) mice indeed restores some of the virulence-associated defects of nonpigmented plague strains, thereby enabling the pathogenesis of plague disease. Our data suggest further that the rV10-2 subunit vaccine may protect individuals with hereditary hemochromatosis against plague. Thus, subunit vaccines, but not live-attenuated vaccines, should be used to protect white populations against plague epidemics.

Moalem et al proposed that the high frequency of the HFE C282Y allele among white individuals [29] may be based on epidemic selection during medieval plague outbreaks in Europe [45]. Because HFE C282Y is associated with decreased iron levels in macrophages, the hypothesis by Moalem and colleagues predicts that, at least early in life, immune cells of individuals with hereditary hemochromatosis may gain the advantage of improved clearance for bacterial pathogens. Similar predictions were fulfilled in an analysis involving mice with a loss-of-function ferroportin mutation, which showed that reduced intracellular iron levels enable macrophage-mediated clearance of intracellular pathogens [46]. Future work will need to examine whether a similar phenomenon occurs for HFE C282Y mutant mice. Nevertheless, we did not observe increased resistance among 6–8-week-old Hjv−/− mice to challenge with fully virulent plague bacteria.

Notes

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