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The realities of biodefense vaccines against *Rickettsia*

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Abstract

Rickettsia prowazekii, *R. rickettsii*, *R. conorii*, and *R. typhi* are serious biologic weapon threats because of high infectivity of low dose aerosols, stable small particle aerosol infectivity, virulence causing severe disease, difficulty in establishing a timely diagnosis, ineffectiveness of usual empiric treatments, potential for engineered complete antimicrobial resistance, lower level of immunity, availability of the agents in nature, and feasibility of propagation, stabilization, and dispersal.

Infection induces long-term immunity, killed rickettsial vaccines stimulate incomplete protection, and a live attenuated mutant stimulates strong immunity but reverts to virulence. Prospects for rational development of a safe, effective live attenuated vaccine are excellent.

Keywords

Biodefense; Vaccines; *Rickettsia*; Weapons

1. Introduction

1.1. Definition of *Rickettsia*

The genus *Rickettsia* comprises a group of genetically closely related, small ($0.3\text{--}0.5 \times 0.8\text{--}1.0 \mu\text{m}$) obligately intracellular bacteria that reside in the cytosol of the host cells and in an arthropod host as at least a part of their ecologic niche [1]. Rickettsiae have undergone evolutionary genome reduction (slightly greater than 1 Mb) as a result of the loss of functions that are provided by the host, including genes encoding enzymes for synthesis of nucleotides, amino acids, and lipids and sugar metabolism [2].

1.2. Characteristics of *Rickettsia* that make them a serious threat as biologic weapons

There are four *Rickettsia* species that frequently cause incapacitating, life threatening illness: *Rickettsia prowazekii*, *R. rickettsii*, *R. conorii*, and *R. typhi*. *R. prowazekii* was the first biologic weapon developed by the Soviet Union during the 1930s [3]. It was produced in powder and liquid forms for use as an aerosol. In the 1930s and 1940s, Japanese military scientists conducted biologic warfare research and field and human testing in northeastern China that included typhus as a biologic weapon [4]. Rickettsiae possess numerous properties that are characteristic of potential biologic weapons, namely high infectivity by low dose aerosol transmission, stable infectivity as a small particle aerosol, virulence causing severe disease, difficulty in establishing a timely diagnosis, ineffectiveness of usual empiric treatments for the undiagnosed clinical manifestations, potential for engineered

complete resistance to antimicrobial treatment, low level of immunity in the population, availability of the agents in known niches in nature, and feasibility of propagation, stabilization and dispersal by persons with a moderate level of microbiologic skills [5].

Guinea pigs and nonhuman primates are highly susceptible to infection via inhalation of aerosols containing *R. rickettsii*. A dose as few as one inhaled rickettsia can cause infection in guinea pigs [6]. All animals that inhaled at least 80 bacteria developed illness, and 75% died. A dose of *R. rickettsii* only 1.5 times that required to cause lethal infection of an embryonated chicken egg after yolk sac inoculation can establish infection in cynomolgus and rhesus monkeys [7]. Among nonhuman primates exposed to aerosol of *R. rickettsii*, 93% became ill and 75% of the sick monkeys succumbed to the infection. Indeed aerosol inhalation is the most efficient route of transmission for monkeys. That humans are also highly susceptible to aerosol transmission of *R. rickettsii* is revealed in the 1976 report by Pike, namely 217 laboratory infections attributed to aerosols compared with only 45 cases of parenteral transmission and 66 cases of transmission from arthropods or animals [8]. *R. rickettsii* is 1000-fold more infectious than the spores of *Bacillus anthracis*. Although these obligately intracellular bacteria are generally considered to be fragile and able to survive for a relatively short period in the extracellular environment, both *R. prowazekii* and *R. typhi* have stable extracellular forms that are present in louse and flea feces, respectively. These rickettsiae appear to remain infectious for a very long time. Similarly rickettsiae can be propagated in cell culture or embryonated chicken eggs and then lyophilized, again remaining stably infectious indefinitely.

The impact of a biologic attack is determined in large part by the proportion of exposed persons who become ill and the severity of the illness. Each infectious disease has its particular fraction of infected persons who develop clinical illness. Some infectious agents result in asymptomatic infection in a substantial percentage of infected persons. e.g., *Coxiella burnetii*, 60%; *Brucella*, 60–90%; *Burkholderia pseudomallei*, 99.9%). *R. prowazekii*, *R. rickettsii*, *R. conorii*, and *R. typhi* appear to cause symptomatic disease in 100% of infected persons. Case fatality ratios, which ought to be a very strong factor in assessing the need for a vaccine against a biologic threat, should be calculated based on the number of deaths in the sum of symptomatic and asymptomatic infections. Because of the potential for engineered antimicrobial resistance, the case fatality ratio should be that of patients that do not receive effective antimicrobial treatment. Historic case fatality rates for Rocky Mountain spotted fever (RMSF) range as high as 80–90% in reports from the first half of the twentieth century in the American West and in Brazil. Currently in Brazil the case fatality rate as high as 40% in spite of the availability of effective antibiotics. It has been hypothesized that some strains of *R. rickettsii* are more virulent than others [9]. Overall in the United States during the preantibiotic era, the case fatality rate for RMSF was 23%. The severity of *R. prowazekii* infections has varied in different settings, as high as 60% in populations suffering from extreme poverty, poor nutrition, alcoholism, exposure to cold, war, and natural disasters and as low as 0% in cases of flying squirrel-associated infections of the contemporary United States population. Most likely 15% of untreated cases of typhus would die under most conditions. Case fatality ratios of 4% for *R. conorii* infections and 1 % for *R. typhi* infections might actually be higher in some populations. Thus one could wonder why *R. rickettsii* is not a Category A agent with similar severity as pneumonic tularemia and why *R. conorii* and *R. typhi* are not at least Category B agents. They have higher case fatality ratios than Rift Valley fever virus, which is Category A, and *C. burnetii*, *B. pseudomallei*, and Venezuelan equine encephalitis virus in Category B. Another factor in determining the impact of an infection is the level of immunity in the general population. The contemporary United States population as judged by the prevalence of antibodies to *Rickettsia* is highly vulnerable to rickettsial infections.

The effectiveness of antimicrobial treatment of rickettsial infection spread by a bioterrorist is compromised by the difficulty of establishing a timely diagnosis. Patients present with fever, headache, myalgia, and frequently also nausea and vomiting. These signs and symptoms could represent a myriad of diseases [10]. A rash that may trigger consideration of a diagnosis of rickettsiosis appears later in the illness or not at all in some cases. Moreover, recognition of the rash can be very difficult in darkly pigmented skin. Epidemiologic clues to the diagnosis such as seasonal and geographic exposure to arthropods (ticks, fleas, body lice) would be absent in aerosol exposed persons. Early empiric treatment with beta-lactam or aminoglycoside antibiotics has no effect on rickettsiae. Essentially every person who dies of a rickettsial disease has been treated with one or more ineffective antibiotics. The drug of choice for rickettsiosis is a tetracycline, and the second line drug is chloramphenicol. Selection of rickettsiae for resistance to chloramphenicol has been published [11], and a reliable source in Russia has reported the development of a strain of *R. prowazekii* that is resistant to tetracycline. It is entirely feasible to create strains of *Rickettsia* that are resistant to both tetracyclines and chloramphenicol by electroporation of resistance plasmids and selection in antibiotic-containing medium. An attack with *Rickettsia* that are universally resistant to antimicrobial treatment is a real possibility.

All of these organisms are present in known niches in nature, can be obtained by collection of these natural sources and inoculation of animals that are available in pet stores, propagated in embryonated chicken eggs purchased from a hatchery without concern for biosafety other than doxycycline for treatment of accidental infection, and stabilized using a purchased lyophilizer.

1.3. A Comparison of vaccine-stimulated immunity to *Rickettsia* and mechanisms of immunity to a primary rickettsial infection

Recovery from a rickettsial infection confers very strong long-lasting protective immunity against subsequent reinfection. Many experiments have documented this principle in infected animals, and anecdotal and limited experimental evidence supports it in humans. Thus development of a vaccine against rickettsiosis is quite possible.

Two types of vaccines against rickettsial infections have been developed and used in humans, whole killed bacteria and live attenuated rickettsiae. In 1924 Spencer and Parker produced a vaccine against RMSF by infecting ticks by allowing them to feed on rickettsemic guinea pigs [12]. Rickettsiae grew in the tick tissues, were harvested, and then killed by phenol and formaldehyde. This vaccine did not prevent infection, but the case fatality rate was reduced dramatically in vaccinees. Required booster immunizations produced substantial local reactions. After a method of cultivating rickettsiae in the yolk sac of embryonated chicken eggs was developed by Cox, this safer easier method was adopted for killed *R. rickettsii* vaccine production. Both of these vaccines were tested in humans in 1973, and all of the vaccinees developed illness after a prolonged incubation period and were treated [13]. In 1970 USAMRIID developed a chick embryo fibroblast cell culture propagated, formalin-killed, sucrose density centrifugation-purified *R. rickettsii* vaccine [14]. When tested in humans in 1983, the vaccine protected 25% of the volunteers from RMSF with lower fever and more rapid response of constitutional symptoms to treatment with tetracycline in vaccinees who developed illness [15]. Killed *R. prowazekii* vaccines were produced by Weigl from the intestines of intrarectally inoculated lice and by Casteneda from the lungs of rabbits infected intranasally. The killed *R. prowazekii* vaccine produced in embryonated chicken egg yolk sacs modified the infection to a milder form and protected U.S. soldiers in zones where there were severe epidemics of louse-borne typhus in the civilian population during World War II.

Although it is apparent that killed *Rickettsia* vaccines are inadequate, at least one lesson can be drawn from the Cox vaccines. The potency tests required by the Food and Drug Administration for release of production lots of these vaccines assayed antibodies to *R. rickettsii* or *R. prowazekii* by a particular cumbersome method, the titer of neutralization by the sera of immunized guinea pigs of rickettsial “toxicity” to mice [16]. This approach utilized a curious animal model that does not represent rickettsial infection, the death of mice inoculated intravenously with an extremely high dose of rickettsiae in less than 24 h. However, contemporary knowledge reveals that the rickettsial antigens that were critical to neutralizing this effect are conformational epitopes of outer membrane proteins (Omp) A and B for *R. rickettsii* and OmpB for *R. prowazekii* [17]. Passively administered antibodies to OmpA or OmpB protect guinea pigs against *R. rickettsii* and protect even SCID mice against a lethal infection with a closely related organism, *R. conorii* [18–20]. Thus, these antibodies are not only correlates of protection but also a protective mechanism.

During World War II a human isolate of *R. prowazekii* was obtained and passaged many times in embryonated eggs, resulting in a fortuitous mutant of low virulence (E strain), which was evaluated as a vaccine in field trials in South America in the 1950s and in Burundi in the 1960s [21]. In a 14-month period after immunization, 94% of subjects were protected from natural epidemic typhus compared with unvaccinated controls [22]. Evaluation of the duration of immunity in volunteers revealed that 96% were protected when challenged 2–36 months afterwards and 83% were protected after 48–66 months. Unfortunately up to 14% of vaccinees developed mild to moderate illness 9–14 days after immunization. That these symptoms were likely owing to reversion to virulence was determined by the development of stable enhancement of virulence of E strain after a few passages in guinea pigs or mice [23]. The isolation of a virulent revertant (Evir strain) allowed subsequent determination that attenuation resulted from a point mutation of Rp028/Rp027 resulting in frameshift and absence of expression of the S-adenosylmethionine-dependent methyltransferase gene [24]. Reversion of the mutation to wild type occurred in Evir strain in which S-adenosylmethionine-dependent methyltransferase is expressed. Correspondingly OmpB of attenuated E strain is hypomethylated, and OmpB of virulent revertant and wild type *R. prowazekii* contains more N^CMe₃-lysine and less N^C-Melysine [25].

Recovery from infection with live rickettsiae is mediated by the innate immune response of TLR-4, dendritic cells, NK cells, endothelial cells, cytokines including IFN- γ , TNF- α , and IL-12, and activated intracellular rickettsicidal mechanisms mediated by nitric oxide (NO), reactive oxygen species, and indoleamine 2,3-dioxygenase and adaptive immunity [26–35]. The most important components of adaptive immunity are cytokine-activated intracellular killing of rickettsiae within the major target cells, endothelium [36,37]. In human endothelial cells, IFN- γ , TNF- α , IL-1 β , and CCL5 combine to activate inducible NO synthase production of rickettsicidal NO and hydrogen-peroxide-mediated rickettsial killing [36,38]. Experimental studies of an inbred mouse model of *R. conorii* infection demonstrated the greater importance of immune CD8⁺ T cells than CD4⁺ T cells and the crucial role of CD8⁺ cytotoxic T cell activity in the clearance of infection [39–41]. Humoral immunity did not play an important role in recovery from infection as antibodies to OmpA and OmpB did not appear until after the animal was well [19].

Two studies of crossprotection further support the concept that although antibodies are a correlate of vaccine-induced immunity other immune mechanisms also contribute to protective immunity. Mice immunized with a sublethal infection with the relatively distantly related *R. australis* or *R. conorii* survived infection with an ordinarily lethal dose of the heterologous organism [42]. In contrast, investigation of the Food and Drug Administration potency test, antibody-mediated neutralization of the mouse toxicity phenomenon, revealed

no crossprotection. In another investigation of crossprotection between even more distantly related *R. typhi* and *R. conorii*, which have minimal serologic crossreactivity, crossprotection against ordinarily lethal doses was observed, and adoptive transfer of T lymphocytes revealed that crossprotection was cell mediated [43]. The genomes of *R. conorii* (1374 genes) and *R. typhi* (only 838 genes) share 791 genes, at least some of which likely stimulate crossprotective CD8+ and CD4+ T lymphocytes [2].

A tentative conclusion is that strongest protection would be provided by stimulating antibodies to the conformational epitopes of OmpA (present in pathogenic *Rickettsia* except *R. prowazekii* and *R. typhi*) and OmpB (present in pathogenic *Rickettsia*) and stimulation of memory CD8+ and CD4+ T cells that recognize the strongest shared antigens.

1.4. Which is the better choice, a sub-unit or a live attenuated rickettsial vaccine?

The ideal rickettsial vaccine would provide solid, lifelong resistance to disease caused by *R. prowazekii*, *R. rickettsii*, *R. conorii*, and *R. typhi* and cause no harm or discomfort in all populations of humans. A subunit vaccine that stimulated persistent antibodies against the conformational antigens of OmpA of *R. rickettsii* and *R. conorii* and of OmpB of all four organisms would be adequate. If strong CD8+ cytotoxic T cell memory responses to antigens recognized by all humans were also induced, an excellent subunit vaccine candidate would be available. Among the challenges would be identification of the appropriate CD4+ and CD8+ T cell antigens and a means to stimulate long-lasting immunity. Recombinant vaccines against OmpA and OmpB have stimulated protection against *R. rickettsii* and *R. conorii*, and native OmpB vaccine has protected against *R. typhi* [44–49]. These proof-of-principle studies indicate that further pursuit of this approach is warranted.

For biodefense purposes it is unlikely that a rickettsial vaccine would be administered to the general public until after one or more terror-inducing attacks had occurred. At that point a large part of the populace would want effective protection, which is more likely to be provided by a live attenuated vaccine than a subunit vaccine. Inactivation of the S-adenosylmethionine methyl transferase gene by homologous recombination removal of a substantial part of the coding sequence could produce an effective non-reverting E strain-like vaccine. Inactivation of the phospholipase D gene of *R. prowazekii* resulted in a mutant that is attenuated for guinea pigs and induces protective immunity [50]. Further rational investigation of potential virulence genes is expected to identify attenuating mutations that singly or in combination will provide vaccine candidates to be tested in rodents and nonhuman primates for safety and efficacy and ultimately in humans for safety. If the goal of a cross-protective vaccine against all *Rickettsia* is achieved, there will be opportunities for its use in tropical medicine, in epidemics of typhus, and for travel medicine as well.

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