Construction and Preliminary Immunobiological Characterization of a Novel, Non-Reverting, Intranasal Live Attenuated Whooping Cough Vaccine Candidate

Cornford-Nairns, R.¹, G. Daggard¹, and T. Mukkur¹,²*

¹Department of Biological and Physical Sciences, University of Southern Queensland, Toowoomba, 4350 Queensland, Australia
²School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Bentley Campus, Perth 6102, Western Australia, Australia

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We describe the construction and immunobiological properties of a novel whooping cough vaccine candidate, in which the aroQ gene, encoding 3-dehydroquinase, was deleted by insertional inactivation using the kanamycin resistance gene cassette and allelic exchange using a Bordetella suicide vector. The aroQ B. pertussis mutant required supplementation of media to grow but failed to grow on an unsupplemented medium. The aroQ B. pertussis mutant was undetectable in the trachea and lungs of mice at days 6 and 12 post-infection, respectively. Antigen-specific antibody isotypes IgG1 and IgG2a, were produced, and cell-mediated immunity (CMI), using interleukin-2 and interferon-gamma as indirect indicators, was induced in mice vaccinated with the aroQ B. pertussis vaccine candidate, which were substantially enhanced upon second exposure to virulent B. pertussis. Interleukin-12 was also produced in the aroQ B. pertussis-vaccinated mice. On the other hand, neither IgG2a nor CMI-indicator cytokines were produced in DTaP-vaccinated mice, although the CMI-indicator cytokines became detectable post-challenge with virulent B. pertussis. Intranasal immunization with one dose of the aroQ B. pertussis mutant protected vaccinated mice against an intranasal challenge infection, with no pathogen being detected in the lungs of immunized mice by day 7 post-challenge. B. pertussis aroQ thus constitutes a safe, non-reverting, metabolite-deficient vaccine candidate that induces both humoral and cell-mediated immune responses with potential for use as a single-dose vaccine in adolescents and adults, in the first instance, with a view to disrupting the transmission cycle of whooping cough to infants and the community.

Keywords: Bordetella pertussis, aroQ, live attenuated pertussis vaccine, cell-mediated immunity induction, antibody response, protection against pertussis

Whooping cough is the major cause of vaccine-preventable deaths today, with WHO estimates of 40–50 million cases and approximately 297,000–409,000 deaths each year worldwide [1, 7, 8, 15], the majority of them being in developing countries although some countries are currently recording increases in the incidence of whooping cough despite high vaccine coverage [39]. A killed whole-cell pertussis vaccine, generally given in combination with diphtheria and tetanus toxoids, has been available in many countries for over 40 years, and although its use seems to have controlled pertussis epidemics, concerns over the reactogenicity, ranging from high fever, persistent crying, pain at the site of injection, and possible, albeit rare, neuropathic manifestations [12, 20, 30, 33, 36], steered the research towards development of the currently marketed acellular pertussis vaccines given in combination with tetanus and diphtheria toxoids (DTaP). Although the introduction and widespread use of the pertussis vaccines caused a dramatic reduction in the incidence of whooping cough, it has risen recently despite high vaccine coverage in developed countries such as Australia, The Netherlands, and the United States despite high levels of immunization rates [http://cdc.gov/pertussis/outbreaks.html; 14]. In Australia, pertussis has been endemic since 1993 with notifications rising from 1.8/100,000 in 1991 to a peak of 156.9/100,000 in 2010 despite a high rate of vaccine coverage [29].

Children under the age of 2 and up to the age of 5 years are highly susceptible to whooping cough. More recently, however, pertussis has re-emerged even in vaccinated populations, confirming that pertussis is not only a childhood
disease but is also highly prevalent in adults [39, 46], the latter being accepted now as the major reservoir of infection for the majority of the pertussis cases in infants and young children. This has stimulated interest in the development of an alternative vaccine that can also be used safely in the adolescent and adult population. Several reasons offered to explain the increasing incidence of this disease syndrome in adolescents and adults include better diagnosis, cyclic variation in disease patterns, waning of vaccine-induced immunity in adolescents and adult over time due to the limited protection offered by the currently used vaccines [24], loss of vaccine efficacy due to the emergence of new B. pertussis strains [25] producing high levels of pertussis toxin (a key virulence factor) [45], and lack of compliance with the recommended vaccination schedule because of the fear of side reactions [12, 19, 33, 34, 36]. Therefore, it is important not only to characterize the newly emerging strains with respect to overproduction of all significant virulence antigens but also to develop alternative vaccine candidates that can be delivered by non-invasive routes (e.g., by oral, intranasal, or epidermal/cutaneous routes) and with potential to impart longer term protection that was offered by the currently marketed acellular pertussis vaccines, an approach divergent from the practices/formulations currently being promoted by most of the major global vaccine manufacturing enterprises.

It is apparent now that the protective efficacy of the acellular vaccine formulations is short to medium, given the recent introduction of new combined acellular vaccine formulations containing reduced antigen content, dTap or Tdap depending upon the vaccine manufacturer, for use in adolescents [26]. To overcome potential side reactions ranging from extensive swelling of the injected limb, which may occur in a concerning percentage of children vaccines receiving booster vaccinations, particularly post-primary immunization with three doses of DTaP, one alternative suggested has been either to reduce the number of booster shots with ensuing reduced levels of immunity or to find a replacement adjuvant, which unlike alum favors the induction of Th1 responses that have been proposed to be responsible for long-term protection against whooping cough [24]. However, as compared with the DTaP, DTwP has been reported to be more protective despite inducing lower antibody titers to pertussis toxin, as judged by intracerebral and lung clearance experiments, leading to the conclusion that cell-mediated immunity may play a crucial role in eliminating bacteria that escape the onslaught of the humoral defence mechanisms in the early phase of infection [2, 17].

An alternative vaccine development approach involving intranasal or parenteral administration of biodegradable particle-encapsulated B. pertussis antigens (PTxoid, FHA, PRN) to mice either induced CMI (Th1) or antibody (Th2) responses that yielded protection that was no better than that induced by the antigens administered in solution [4, 40]. Given the relatively recent communication suggesting Th1/Th17-mediated host immunity against pertussis [28], it is apparent that the exact nature of immunity against infection with B. pertussis is still not completely understood and requires further elucidation.

Mice vaccinated with PT-deficient live B. pertussis were reported to be protected against challenge with virulent B. pertussis [22]. In another study, an aromatic-dependent mutant (aroA) of B. pertussis [35] was reported to persist in the lungs of mice for a short period of time (4 days at reasonable numbers), thus casting doubt on its ability to stimulate the cell-mediated immunity (CMI) required for long-lasting protection. This result was unexpected, given previous reports regarding the success of the aroA mutant of Salmonella species [13, 27] as a successful vaccine. On the other hand, the aroA mutants of Shigella species were found to be poorer vaccines than the aroD deletion mutants of the same species [43]. It was suggested that the development of live attenuated whooping cough vaccine delivered by the nasal route, so as to mimic the natural route of infection, leading to induction of long-lasting immunity, represented an ideal solution that deserved serious attention [18]. More recently, a live attenuated B. pertussis mutant strain, in which PT was genetically detoxified, the dermonecrototoxin (DNT) gene deleted, and B. pertussis ampG replaced by E. coli ampG to reduce its toxic activity, was reported to induce protection in young mice after a single nasal administration [23] and reported to be safe in adult interferon-γ receptor deficient adult mice, although the vaccine candidate survived in lungs of vaccinated mice for as long as the wild-type strain. We report the development of an aromatic-dependent (aroQ) B. pertussis mutant that was found not only to induce B. pertussis-specific antibody response and cell-mediated immunity in mice immunized with a single dose of this vaccine candidate by the intranasal route, but was also protective against challenge infection with virulent B. pertussis. Upon challenge infection of immunized mice with virulent B. pertussis, both antibody and CMI responses were significantly or substantially enhanced, respectively, imparting protection against the challenge infection.

**Materials and Methods**

**Bacterial Strains, Plasmids, Media, and Growth Conditions**

The bacterial strains used in this study were virulent Bordetella pertussis, ATCC 9340 (Microdiagnostics, Brisbane, Australia), Streptomycin-resistant [Sm³] B. pertussis (obtained through courtesy of Dr. A. Weiss, University of Cincinnati, OH, USA), aroD E. coli mutant (obtained through courtesy of Dr. Naresh Verma, Australian National University, Canberra, Australia), E coli DH5α [F′ λ80dλacZM15 Sm(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK−,mT−) phoA supE44 lacI− gyrA96 relA1; Invitrogen Life Technologies],