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Vaccines against intracellular bacterial pathogens

Richard W. Titball

School of Biosciences, University of Exeter, Exeter, EX4 4QD Devon, UK

There is a long history of remarkable success in developing vaccines against bacteria that are extracellular pathogens. In general, the development of vaccines against intracellular bacterial pathogens has proven to be more challenging. Typically, such vaccines need to induce a range of immune responses, including antibody, $CD4^+$ and $CD8^+$ T cell responses. These responses can be induced by live attenuated vaccines, but eliciting these responses with non-living vaccines has proven to be difficult. The difficulties appear to be related partly to the problems associated with the identification of protective antigens and partly with the difficulties associated with inducing $CD8^+$ T cell responses.

The need for vaccines against extracellular and intracellular bacterial pathogens

During the late 19th century and the first part of the 20th century a wide range of vaccines against bacterial infectious disease were devised [1]. Many of these vaccines were developed on an empirical basis, with little understanding of the basis of disease. By today's standards, most of these vaccines would be considered to be crude, and it is highly unlikely that they would meet the current requirements for vaccine licensing. Nevertheless, these vaccines have played major roles in the control of infectious diseases, including diphtheria, whooping cough and tetanus, and the derivatives of some of these vaccines are still in use today. More recently, effective vaccines against some serogroups of *Neisseria meningitidis* [2] and *Streptococcus pneumoniae* [3,4] have become available.

All of these bacteria adopt a predominantly extracellular lifestyle and protection can be induced by immunising with discrete components (subunits) from these bacteria. While there are a number of extracellular bacterial pathogens for which effective vaccines are still not available, arguably the greatest challenge facing the community nowadays is the development of vaccines against pathogenic bacteria that have a predominantly intracellular lifestyle (Table 1). This need is partially driven by the heightened level of interest in devising vaccines against biowarfare agents. For example, the candidate biowarfare agents *Brucella suis*, *Brucella melitensis*, *Brucella abortus*, *Francisella tularensis*, *Burkholderia pseudomallei* and *Burkholderia mallei* are all intracellular pathogens. Outside of the biodefence field, intracellular pathogens such as *Mycobacterium tuberculosis* and *Salmonella enterica* remain priority targets for the development of vaccines for use in both humans and in animals.

Vaccines against extracellular and intracellular bacterial pathogens may need to elicit different types of immune response

The remarkable success of vaccines against extracellular pathogenic bacteria can be attributed to two related factors. Firstly, protective immunity is elicited towards either a single antigen or a limited number of antigens. These antigens can be isolated from bacteria that are grown *in vitro*. Secondly, protective immunity is predominantly dependent on the induction of antibody responses to these antigens. Often, the antibody responses are directed towards toxins, and neutralising antibody is able to bind to these toxins and physically block their interaction with host cells. For example, in the case of diphtheria, tetanus and anthrax vaccines, the antibody that is raised against the vaccine is able to neutralise the diphtheria, tetanus and anthrax toxins, respectively [5,6]. The nature of protective immunity induced by pertussis vaccines is less well understood, but neutralising antibody directed against the pertussis toxin appears to play a key role [7].

Antibody directed against other pathogens, such as the meningococci, appears to have a bactericidal effect, mediated via activa-

E-mail address: R.W.Titball@exeter.ac.uk.

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TABLE 1

Pathogens for which effective vaccines are currently a focus of research activity

Bacterial pathogens with a predominantly extracellular lifestyle	Bacterial pathogens with a predominantly intracellular lifestyle
Yersinia pestis	*Brucella suis*
Bacillus anthracis	*Brucella melitensis*
Vibrio cholerae	*Brucella abortus*
***Neisseria meningitidis* B**	*Francisella tularensis*
Helicobacter pylori	*Burkholderia pseudomallei*
Streptococcus pneumoniae	*Burkholderia mallei*
Haemophilus influenzae	*Mycobacterium tuberculosis*
	Salmonella enterica

tion of the classical pathway of complement activation [2,8,9]. Protective immunity against *S. pneumoniae* infection appears to be mediated primarily by opsonising antibodies directed against the polysaccharide capsule [3,4]. The opsonised bacteria are then ingested and killed, mainly by alveolar macrophages [10]. Vaccine-induced protection against *Haemophilus influenzae* appears to be mediated by both opsonising and bactericidal antibodies [11]. Therefore, in the case of many extracellular pathogens protective immunity can be explained by the presence of antibodies that are able to neutralise toxins or that have opsonising or bactericidal activities.

In the case of intracellular pathogens, the nature of protective immunity is much less clearly defined. Intracellular pathogens, by their very nature, cause disease after invading and growing in host cells. This generally involves an array of virulence factors acting in concert. Therefore, unlike extracellular pathogens, it is unusual to identify a single gene product, such as a toxin, which plays an overriding role in the pathogenesis of disease. Additionally, an often-stated paradigm is that cellular immunity is crucial for protection against intracellular bacterial pathogens [12]. Antigens that play roles in the pathogenesis of disease caused by intracellular pathogens are likely to have limited visibility to the immune system and are generally not good targets for the antibody-mediated protection. However, there are number of examples where antibodies have been shown to afford some protection against intracellular bacterial pathogens [12,13]. Possibly these antibodies interact with the pathogen before it enters target cells, either shortly after infection, or during cell-to-cell spread of the pathogen. Or, these antibodies might be able to cross the host cell membrane and could be active in the cytosol. Alternatively, if antigens derived from bacteria are presented on the host cell surface then antibody dependent cell killing might occur [12,13]. Although antibodies can provide protection against some intracellular pathogens, at least in small animal models of disease, it is also clear that the level of protection that they alone offer is relatively low. For example, antibodies can protect against low but not high virulence strains of *F. tularensis* [14] or can provide protection against low challenge doses of *B. pseudomallei* given by the intraperitoneal route, but not given by the aerosol route of infection [15,16].

Therefore, although antibodies might contribute to protection, the consensus is that cellular immune responses, and especially those involving $CD4^+$ and $CD8^+$ T cells, are crucial for protective immunity against intracellular pathogenic bacteria [17–19]. These different T cell types play distinct and complementary roles in protective immunity. For example, $CD4^+$ T cells produce a range of cytokines that orchestrate the immune response and may activate host cells, such as macrophages, to kill the pathogen [19]. $CD8^+$ T cells (cytotoxic T cells) are able directly to kill infected cells [19]. The role of T cells in protective immunity to *Listeria monocytogenes* is elegantly demonstrated by the finding that $CD8^+$ T cells that recognise a single epitope can protect against infection [20]. However, for the majority of intracellular pathogens, the protective response is much more complex. For example, while $CD8^+$ T cells are the major T cell type involved in protective immunity to brucellosis [21,22], it is clear that an, as yet, poorly defined range of epitopes are recognised [21]. Both $CD4^+$ and $CD8^+$ T cells have been shown to play key roles in protective immunity to *M. tuberculosis*, *Mycobacterium paratuberculosis* [23].

More recently γδ T cells have been implicated in protective immunity [24]. While $CD4^+$ and $CD8^+$ T cells recognise peptides derived from bacterial proteins, γδ T cells often recognise non-peptidic phosphorylated isoprenoid pathway metabolites, referred to as phosphoantigens [24,25]. Elevated levels of γδ T cells appear to be a feature of infection with many intracellular pathogens [26–29]. Although their function is poorly defined, activated γδ T cells might contribute to protection by their direct cytolytic activity and their abilities to produce inflammatory cytokines [30]. The direct cytolytic activity has been attributed to the production of anti-microbial peptides such as LL37 [26].

Overall, it is quite clear that protection against extracellular and intracellular bacterial pathogens can be dependent on quite different immune responses. However, the often-stated view that protection against extracellular bacteria is dependent on antibody, while protection against intracellular bacteria is dependent on cellular responses, while broadly true, is also an oversimplification. In particular, it is apparent that protection against intracellular bacteria is dependent on the induction of a range of responses that in concert can provide protection [31].

Different approaches to the development of vaccines for intracellular and extra cellular pathogens

The finding that different immune responses are required for protection against different pathogens is a key factor when considering the type of vaccine that should be developed. For example, vaccines that are based on killed whole cells, or on isolated protein or polysaccharide fractions of cells, can induce good antibody responses, but are generally poor at inducing cellular immu-

TABLE 2

Comparison of the immunogenic potential, clinical safety and producibility of the main types of bacterial vaccine

	Killed whole cell	Subunit	DNA vaccine encoding subunit	Live vector delivered subunit	Live attenuated
Immunogenic potential					
Antibody responses	+++	+++	+	++	++
$CD4^+$ T cell responses	+	+	++	++	++
$CD8^+$ T cell responses	–	–	++	++	+++
Safety					
Tolerability	+	+++	++	++	++
Transmissibility	–	–	–	++	+++
Safety in immunocompromised Individuals	++	+++	+++	+	+
Producibility					
Cost	+	++	+++	+++	+
Potential for needle free delivery	–	+	+	++	+++

nity [32,33]. Consequently, killed cells or subunits are the mainstays of vaccines against extracellular pathogens. Conversely, live attenuated mutants induce a broad repertoire of immune responses including strong $CD8^+$ T cell responses and are often the best route to an effective vaccine against intracellular pathogens (Table 2).

However, the selection of an appropriate vaccine goes beyond the matching of the desired to the realised immune response. Although live vaccines against intracellular pathogens often provide the appropriate immune response, they are generally less favoured than non-living vaccines. Often, the path to development of a new, live attenuated vaccine is complicated. For example, in the case of *S. enterica* vaccines, a problem with transient bacteraemia only became apparent during human trials and necessitated the identification of an additional mutation to block this event [34]. Of greatest concern, live vaccines may not be safe in immunocompromised individuals [35,36]—an increasing population in society. Similar concerns over the safety of the vaccine apply to the delivery or vaccine subunits in live vaccine vectors, such as vaccinia virus, adenovirus or *Salmonella*, which may not be safe in immunocompromised individuals [37–39].

To attempt to resolve some of these problems alternative approaches have been described that aim to capture the ability of live vaccines to induce cellular immune responses against a range of antigens but without any of the problems of devising suitable attenuated mutants that are safe. The so-called killed but metabolically active (KBMA) bacteria are generated by removing UV repair genes and then photochemically inactivating the bacteria with psoralen and long-wavelength ultraviolet light [40]. The treated bacteria are unable to form colonies on growth media but are capable of synthesizing and secreting proteins. KBMA mutants of *L. monocytogenes* are reportedly capable of inducing $CD4^+$ and $CD8^+$ T cell responses [40]. The wider application of KBMA technology to other intracellular pathogens has yet to be realised.

Against this background, one of the current and major, areas of research activity involves identifying ways in which subunits can be delivered to evoke not only humoral, but also cellular immunity. One approach involves the use of naked DNA delivery systems. Initially heralded as a breakthrough in vaccine delivery, the immunisation of mice with naked DNA evoked strong humoral and cellular responses. Disappointingly, in non-human primates and in humans, naked DNA vaccines have evoked only weak responses [19]. However, there are signs that naked DNA vaccines are moving towards maturity. In 2007, the first commercially available naked DNA vaccine for the prevention of West Nile Fever in horses was licensed.

An alternative approach to the induction of cellular immunity by vaccine subunits involves their formulation with adjuvants capable of promoting this type of response. Many adjuvants function by promoting a 'depot effect' that results in the slow release of antigen from the vaccination site. However, the induction of cellular responses requires adjuvants to activate the appropriate elements of the innate immune system. Often this is achieved because the adjuvant binds to Toll-like receptors and nucleotide-binding oligomerisation domain (NOD) receptors [41]. However, the activation of these pathways is often associated with inflammatory responses, which unless carefully controlled would be unacceptable in vaccines destined for use in humans. Although a broad range of experimental adjuvants have been described that are capable of inducing cellular responses [41], these adjuvants are generally not suitable for clinical use because of their reactogenicity. In addition, it is questionable whether any of these adjuvants are capable of promoting strong $CD8^+$ responses, which are often required for protection against intracellular pathogens [42]. Against this background there is currently a high level of interest in developing adjuvants suitable for use in humans [41]. The success of this initiative is likely to be dependent on the separation of the adjuvant and toxic effects. Whether this is achievable is currently open to debate [41]. Like naked DNA vaccines, this field might be led by the veterinary vaccine industry, where a range of adjuvants capable of promoting cellular responses is already approved for use in animals [43].

From adjuvants that activate cells of the innate immune system, one logical line of investigation is to target these cells directly with the antigen of interest. Increasingly, this approach looks feasible. Antigen presenting cells play roles in orchestrating the immune response, and several reports suggest that it may be possible to target subunits towards antigen-presenting cells and, especially, to dendritic cells [44]. This approach has recently been shown to offer promise for the development of *M. tuberculosis* vaccines [45].

The identification of protective subunits for intracellular pathogens remains a major challenge

The formulation of vaccines to promote cellular immunity is one of the challenges facing the research and development community. A second, and possibly greater, challenge is to identify the components of the bacterium that should be included in a vaccine. In the case of *L. monocytogenes*, a response to a single protein (listeriolysin O) is sufficient to provide protection [20]. However, it is clear that this is an exceptional situation. For many Gram-negative intracellular pathogens, immunisation with components such as lipopolysaccharide or proteins can provide limited protection against disease [14,16,46–50]. However, the identification of a single component (or a limited pool of components) that provides high levels of protection when used as an immunogen is an ongoing challenge. It may be the case that for many of these pathogens it is necessary to immunise with a mixture of subunits to elicit a protective response that is comparable to that elicited by a live attenuated vaccine.

Genomic and proteomic data might allow the fast-tracking of vaccine research

Traditionally, empirical approaches to development of vaccines have been employed. However, the success of this approach is often dependent on a large body of data on the pathogenesis of disease and on virulence mechanisms. Therefore, the development of vaccines might be dependent on decades of underpinning research. For many of the pathogens that are the subject of current vaccine programmes, this underpinning information is not available. To allow vaccine research and development programmes to progress in the absence of these data, bioinformatic-based approaches are increasingly used. Ultimately, this approach is driven by the availability of genome sequence data. Broadly, genome sequence information might be used in two ways. It could be used to identify biochemical pathways that could be inactivated to yield rationally attenuated mutants exploitable as vaccines. Secondly, these data could be used to screen genomes rapidly for open reading frames that could encode potential subunits for inclusion in vaccines. Genome-driven vaccine design has been termed reverse vaccinology [51–53].

Conclusion

This review is concerned with technology advances that impact on the development of vaccines against bacterial pathogens, but many of the issues raised apply equally to pathogens such as viruses and parasites. The most effective vaccines against intracellular pathogens are live attenuated mutants—they induce a wide range of responses against a broad range of cellular components. An ongoing challenge is to replace these live attenuated mutants with subunit vaccines. For some intracellular bacterial pathogens this goal is achievable but for others it may not be. For many intracellular pathogens it might be necessary to use combinations of subunits to provide a protective response. However, an added complication is that it might be necessary to induce different types of responses to different subunits. A strong antibody response to antigens such as LPS might need to occur alongside $CD8^+$ T cell responses to others. The technical challenges associated with these programmes are significant.

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