Vaccines, Reverse Vaccinology, and Bacterial Pathogenesis

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Advances in genomics and innovative strategies such as reverse vaccinology have changed the concepts and approaches to vaccine candidate selection and design. Genome mining and blind selection of novel antigens provide a novel route to investigate the mechanisms that underpin pathogenesis. The resulting lists of novel candidates are revealing new aspects of pathogenesis of target organisms, which in turn drives the rational design of optimal vaccine antigens. Here we use the discovery, characterization, and exploitation of fHbp, a vaccine candidate and key virulence factor of meningococcus, as an illustrative case in point. Applying genomic approaches to study both the pathogen and host will ultimately increase our fundamental understanding of pathogen biology, mechanisms responsible for the development of protective immunity, and guide next-generation vaccine design.

Along with improved sanitation and the discovery and use of antibiotics, vaccination is the intervention that has had the greatest impact on human health and the standard of living in recent history. Vaccines also represent the most cost-effective way of improving health and saving lives (Levine and Lagos 2004). In the past, the development of new vaccines and therapeutics was aided, and largely driven, by increased understanding of the pathogenesis of infectious agents. The antigens used in vaccines do not necessarily have to be virulence factors; however, understanding and interrupting the cycle of infection by directing the immune responses toward key virulence determinants has historically been a successful rationale in eliciting protective immunity. However, the development of vaccines for many pathogens remains elusive, and there is a growing requirement for the fast development of effective vaccines for emerging diseases (Morens et al. 2008). During the last three decades, the vaccine field has been transformed by new technologies such as recombinant DNA and chemical conjugation. More recently, new methods of antigen discovery and design as well as investigation of vaccine responses have been applied, including reverse vaccinology, structural biology, and systems biology (Rinaudo et al. 2009). Genome-based technologies have enabled functionally blind selection of vaccine candidates, and have not only led to the discovery of novel protective antigens but have also revealed new virulence factors of several pathogens. Consequently, the pathogenesis-to-vaccine paradigm has been reversed in several situations, and vaccine development frequently leads to a better understanding of pathogenesis, which has in turn led to novel

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approaches in studying not only the organism itself but also the strategies for the design of more successful vaccines (Fig. 1). The continuing advancement of genome-based technologies will hopefully lead to new vaccines for unmet diseases, as well as increasingly efficient development pathways for these vaccines (Rappuoli et al. 2011).

HOW OUR UNDERSTANDING OF BACTERIAL PATHOGENESIS HAS LED TO NEW VACCINES

Since the first observations of Jenner at the turn of the nineteenth century, that milkmaids exposed to cowpox appeared to be protected from the more severe human smallpox disease, the understanding of pathogenesis has been fundamental to the development of vaccines. Once it became evident in the early 1800s that diseases were caused by microbes, Louis Pasteur started the rational development of vaccines and established the basic rules of vaccinology. Most of the vaccines licensed to date have been developed based on Pasteur's principles of "isolate, inactivate and inject" the causative agent of disease (Plotkin 2009). One could say that they have been developed empirically, with little or no understanding of the complex immunological mechanisms by which they induce protective immunity. However, the strategy was based on a unifying rationale, that by reducing the virulence of the disease-causing organism or by inhibiting its ability to replicate, the consequent

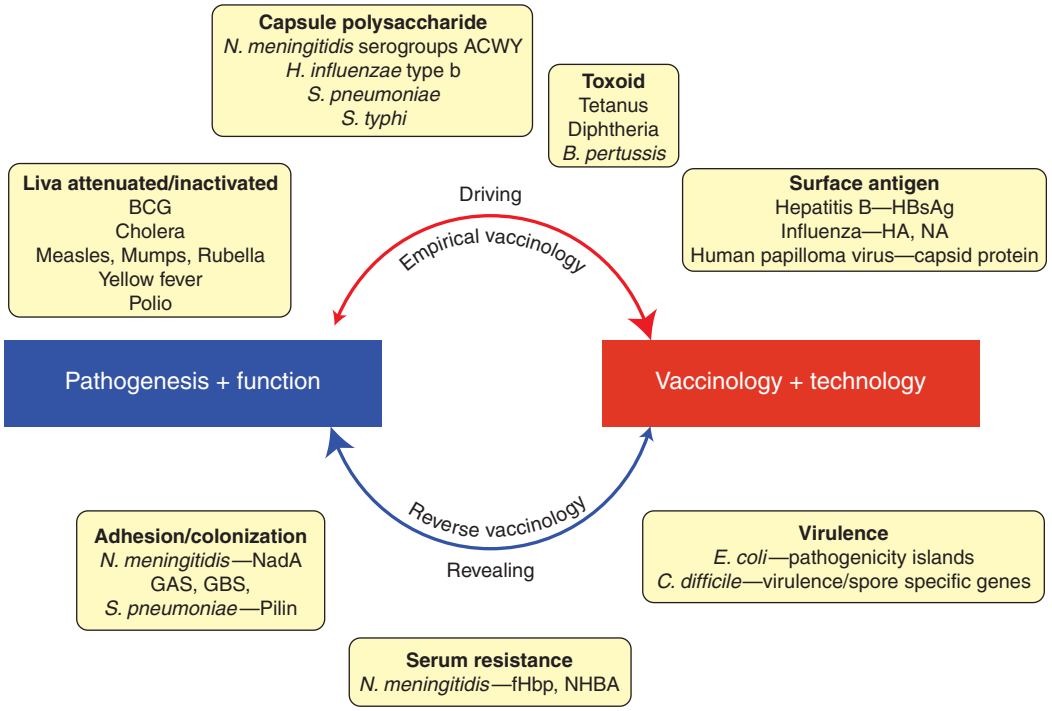

Figure 1. Interactions between microbial pathogenesis and vaccine development. Historically, the understanding of microbial mechanisms of pathogenesis have driven vaccine development, with empirical vaccinology approaches being based on inactivating, attenuating, and/or targeting and interrupting the function of virulence factors, such as capsule polysaccharides, toxins, and other surface proteins. However, with new technologies and vaccine approaches, the focus has shifted to functionally blind antigen discovery based on high-throughput screening of the pathogen's genome and proteome. These approaches have identified many promising vaccine candidates, which have subsequently been found to be involved in the pathogenic process, such as colonization of the host or serum resistance.

attenuation or inactivation robs infectious organisms of their pathogenicity while preserving their immunogenicity. The mechanism of action of vaccines relies entirely on activation of the human body's own protective immunological mechanisms, and live-attenuated or inactivated vaccines basically mimic the kind of protective immunity induced in people who survive a normal infection.

Pasteur applied the principles of "isolate, inactivate and inject" in the late 1800s to both viruses (dried rabies virus isolated from rabbits) and bacteria (heat-inactivated anthrax bacilli). These strategies have been successful for those diseases that result in natural immunity to reinfection, in particular for many viral diseases that have invariant antigens (e.g., smallpox, yellow fever, polio, rabies, mumps, measles, rubella, varicella, herpes zoster, hepatitis A, and Japanese encephalitis) (Rappuoli 2007). Furthermore, inactivated or live-attenuated vaccines against some bacterial infectious diseases have also been developed with varying degrees of success; for instance, Bacille Calmette-Guerin (BCG; live-attenuated *Mycobacterium bovis*) is possibly the most widely used vaccine worldwide and currently the only vaccine available against tuberculosis (Parida and Kaufmann 2010). Whole-cell vaccines against enteric diseases such as typhoid and cholera, and a whole-cell pertussis vaccine are currently licensed and in use, although there is a general need and trend to replace these with newer improved vaccines.

A further development to Pasteur's strategy was undertaken when essential components of the organism, usually virulence determinants, were isolated and inactivated, for use as "subunit" vaccines. This approach was first used for diphtheria and tetanus in which the pathology of the disease is largely owing to the toxins produced by the bacterial pathogen, and initial toxoid vaccines were chemically inactivated toxins isolated from the bacteria (Glenny and Hopkins 1923; Ramon 1924). Similarly, capsule polysaccharides of Gram-negative bacteria are common virulence factors and subunit vaccines based on purified polysaccharides were initially developed for *Streptococcus pneumoniae* in the 1940s (MacLeod et al. 1945), *Neisseria meningitidis* in the 1960s (Gotschlich et al. 1969), *Haemophilus influenzae* in the 1970s (Peltola et al. 1977), and *Salmonella typhi* Vi in the 1980s (Acharya et al. 1987). With advances in new technologies, such as recombinant DNA and chemical conjugation of proteins to polysaccharides, subunit vaccines have advanced notably and are able to provide improved memory responses and increased protection in infants. These vaccines are safe and efficacious, and have also been developed in multivalent forms to provide protection against numerous pneumococcal serotypes and meningococcal serogroups. For the three major causes of bacteremic disease—*H. influenzae* type b (Hib), pneumococci, and meningococci—the use of polysaccharide conjugate vaccines has led to successful control of invasive diseases caused by many serogroups in various countries worldwide (Kelly et al. 2004; Gill et al. 2010; Jefferies et al. 2011).

Genetic inactivation by targeted mutagenesis as well as modern recombinant techniques for the expression and purification of protein-based subunit vaccines have led to the development of safer live-attenuated vaccines and subunit vaccines through rational design. For example, recombinant DNA technology enabled the generation of the detoxified *Bordetella pertussis* toxin (Pizza et al. 1989), and the recombinant hepatitis B surface antigen (HBsAg) (Buynak et al. 1976; Ellis 1990). Ultimately, recombinant DNA technology has formed the basis of a wide range of new platforms for vaccine design and delivery.

Subunit vaccine antigens are usually better tolerated than inactivated or live-attenuated pathogens, and this has led to a push for the development of new and improved vaccines using recombinant techniques. Acellular pertussis combination vaccines, containing between one and five pertussis virulence factors are substantially less reactogenic than the original whole-cell vaccines, and have increasingly replaced whole-cell-containing vaccines in developed countries (Kitchin 2011). However, a limitation of subunit vaccines is that they are generally less immunogenic and often require the addition of an adjuvant to elicit protective immune

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responses (Mbow et al. 2010). Unfortunately, few adjuvants are currently licensed and widely used (e.g., aluminum salts, Alum, that have been used for more than 80 years). However, newer adjuvants (e.g., MF59 [O'Hagan et al. 2011] and AS04 [Garcon et al. 2011]) have been developed, each of which have specific properties designed to enhance the magnitude and quality of the immune response to target specific diseases.

Although the virtual disappearance of diseases like diphtheria, tetanus, pertussis, and invasive *H. influenzae* B are landmarks to the success of vaccines, there still remain many bacterial diseases for which vaccination strategies have so far been unsuccessful. Often this is owing to the hypervariability of their antigens, an incomplete understanding of pathogenic mechanisms, and/or a need for cell-mediated immune responses (Rappuoli 2007). For instance, for those diseases that do not induce immunity after natural infection (e.g., malaria, or respiratory syncytial virus [RSV]) or those that cause persistent or latent infection (e.g., HIV and hepatitis C virus), a vaccine-induced protective immune response must go one step beyond the mechanisms that nature has evolved and vaccine development must go beyond the traditional empirical methodologies.

REVERSING THE PARADIGM: REVERSE VACCINOLOGY APPROACHES LEAD TO INCREASED UNDERSTANDING OF BACTERIAL PATHOGENESIS

With the arrival of whole-genome sequencing, genome-based antigen selection has played a major role in antigen discovery and vaccine design. One approach that has been used to mine pathogenic bacterial genomes has been coined "Reverse vaccinology," and allows the investigation of the complete potential antigenic repertoire of an organism from its genome sequence. Reverse vaccinology involves the cloning and expression of all the proteins in an organism's genome sequence that are predicted in silico to be surface exposed or secreted. Then each protein is screened, through high-throughput immunization, for their ability to elicit antibodies in mice that can kill or neutralize the target organism. The first pathogen addressed using reverse vaccinology was *N. meningitidis* serogroup B (MenB) (Pizza et al. 2000), for which no broadly protective vaccine exists owing to the similarity of its capsular polysaccharide to a self-antigen and the hypervariability of its major outer membrane protein antigens. This approach led to the identification of 29 novel antigens that can elicit bactericidal antibodies against the pathogen in vitro. After successful preclinical studies, the vaccine deriving from the genome approach entered phase I testing in 2002 (Giuliani et al. 2006). In November 2012, the European Medicinal Agency recommended the granting of a marketing authorization for Bexsero, the first vaccine to provide broad coverage against meningococcal serogroup B. This was followed by the approval of the European Commission in January 2013. The three main antigens identified by reverse vaccinology that are formulated as components of the MenB vaccine, *Neisserial* heparin-binding antigen (NHBA), factor H-binding protein (fHbp), and *N. meningitidis* adhesin A (NadA), have all subsequently been implicated as playing important roles in meningococcal virulence (Capecchi et al. 2005; Madico et al. 2006; Schneider et al. 2006; Serruto et al. 2010) (see below for more details on fHbp).

Since the first application of reverse vaccinology, based on the single genome of a MenB strain (Pizza et al. 2000), various advancements have been made to this genomic approach. A multigenome or pan-genome reverse vaccinology approach was applied to Group B streptococcus (GBS) to identify antigens from the extended gene repertoire of the species rather than from a single organism (Maione et al. 2005), and the subtractive reverse vaccinology approach has been used to identify antigens present in pathogenic but not commensal strains of *Escherichia coli* (Rasko et al. 2008; Moriel et al. 2010). Reverse vaccinology has been applied to a wide range of bacterial pathogens and has provided a long list of promising antigens from functionally blind interrogation of their genomes, and the subsequent studies on antigen function are leading to increased understanding of the biology of the pathogens (Fig. 1; Table 1).

Table 1. Microbial virulence factors identified during vaccine development

Organism	Disease	Vaccine approach/genomic-based technology	Antigen/virulence factor	Role	References
Neisseria meningitidis	Meningococcal meningitis and sepsis	Reverse Vaccinology: genome analysis of serogroup B strain Structural Vaccinology: chimeric fHbp engineered based on variant 1 backbone-containing epitopes from variants 2 and 3	Factor H-binding protein (fHbp)	Binding of complement factor H (fH), serum resistance	Pizza et al. 2000, 2008; Giuliani et al. 2006; Madico et al. 2006; Scarselli et al. 2011
		Reverse Vaccinology: genome analysis of serogroup B strain	Neisserial adhesin A (NadA)	Adhesion, colonization	Comanducci et al. 2002; Capecchi et al. 2005
		Reverse Vaccinology: genome analysis of serogroup B strain	Neisserial heparin-binding antigen (NHBA)	Binding of heparin, serum resistance	Serruto et al. 2010
Group B *Streptococcus (Streptococcus agalactiae)*	Sepsis, pneumonia, and neonatal meningitis	Pan-Genome Reverse Vaccinology: genome analysis of 8 GBS strains Comparative Genomics and Genome Hybridization: *Streptococcus pneumoniae*, GAS genomes, 19 GBS strains	Pili (BP-1, BP-2a, AP-1, AP-2a, GBS67)	Adhesion, colonization, biofilm formation, translocation through epithelial cells	Lauer et al. 2005; Maione et al. 2005; Tettelin et al. 2005; Dramsi et al. 2006; Rosini et al. 2006; Pezzicoli et al. 2008; Margarit et al. 2009; Rinaudo et al. 2010; Nuccitelli et al. 2011
Group A *Streptococcus (Streptococcus pyogenes)*	Pharyngitis, necrotizing faciitis, pneumonia, sepsis, arthritis, myositis	Pan-Genome Reverse Vaccinology/ Comparative Genomics: analysis of five GAS strains for pilus encoding (LPXTG motif) genes	Pili (Lancefield antigens)	Adhesion, colonization, biofilm formation	Mora et al. 2005; Abbot et al. 2007; Manetti et al. 2007
Streptococcus pneumoniae	Pneumonia, meningitis, otitis media, sepsis	Reverse Vaccinology/Comparative Genomics: analysis of T4 and INV104 genomes for putative virulence factors and pilus genes	Pili (RrgB, RrgA, RrgC encoded by *rlrA* pathogenicity Islet; PitB, pilus islet 2)	Adhesion, colonization, biofilm formation	Hava and Camilli 2002; Barocchi et al. 2006; LeMieux et al. 2006; Bagnoli et al. 2008; Hilleringmann et al. 2009; Donati et al. 2010

Continued

Table 1. *Continued*

Organism	Disease	Vaccine approach/genomic-based technology	Antigen/virulence factor	Role	References
Escherichia coli	Intestinal (diarrhea) and extraintestinal (urinary tract infection, sepsis, meningitis, hemolytic-uremic syndrome) disease	Comparative/Subtractive Reverse Vaccinology: comparison of genomes of commensal and pathogenic strains Proteomics: analysis of OMV proteins	Pathogenicity islands, pathogen-specific OMPs (e.g., ECOK1_0290)	Adhesion, colonization	Berlanda Scorza et al. 2008; Moriel et al. 2010; Nesta et al. 2012
Staphylococcus aureus	Nosocomial, skin, wound infections, endocarditis, pneumonia, bacteremia, osteomyelitis, toxic shock syndrome	Reverse Vaccinology: genome analysis of eight strains for the presence of sortase substrate genes (based on loss of pathogenesis of sortase mutant) Comparative Genomics: genome analysis of 58 strains for variation of putative surface proteins	Conserved virulence-associated surface proteins (e.g., IsdA, IsdB, SdrD, SdrE)	Pathogenesis in murine renal abscess model	Mazmanian et al. 2000; Stranger-Jones et al. 2006; McCarthy and Lindsay 2010
Clostridium difficile	Nosocomial diarrhea	Comparative Genomics: genome analysis of historic nonepidemic and newly emerged hypervirulent epidemic strains, and evolutionary dynamics of 30 isolates Proteomics: investigation of core spore gene, spore–host interactions	Genetic regions specific for epidemic strain (e.g., flagella biosynthesis and glycosylation proteins) 29 *C. difficile* spore specific proteins	Motility, autoagglutination, sporulation	Stabler et al. 2006, 2009; Lawley et al. 2009; He et al. 2010
Chlamydia trachomatis	Most prevalent sexually transmitted infection worldwide (causes ectopic pregnancy, infertility), trachoma	Reverse Vaccinology: genome analysis Comparative Genomics: genome analysis of five chlamydial species Proteomics: investigation of elementary bodies	Outer membrane complex and elementary body proteins	Host–pathogen interactions	Montigiani et al. 2002; Thorpe et al. 2007; Heinz et al. 2009; Liu et al. 2010

OMV, outer membrane vesicle; OMPs, outer membrane proteins.

Although a vaccine antigen does not have to be a virulence factor, focusing on proteins with a crucial function in bacterial pathogenesis or survival is a useful selection criterion, because antibodies elicited to these antigens may block their function and result in neutralization. For example, the use of protective antigens involved in adhesion is particularly attractive, as there may be important implications for providing herd immunity through reduction of the pathogen's colonization and circulation. Indeed, functional studies of many protective antigens have subsequently revealed them to be novel adhesins involved in colonization of the pathogen (Table 1) (Capecchi et al. 2005; Rosini et al. 2006). The pili of gram-negative bacteria are well-described virulence factors, however, little was known about pili in gram-positive bacteria before their discovery as a result of reverse vaccinology on GBS (Lauer et al. 2005; Maione et al. 2005), and subsequent studies on *Streptococcus pyogenes,* and *S. pneumoniae* (reviewed in Telford et al. 2006). The presence of pili that contain protective antigens in all three principal streptococcal pathogens indicates that these structures play an important role in virulence. Genome-based comparison of pathogenic and nonpathogenic strains has also been applied to enable vaccine targets to be identified on the basis of proteins that are specifically present in pathogenic strains and therefore implicated in virulence. Recently, subtractive reverse vaccinology has been applied to *E. coli* and five out of the nine protective antigens identified are located on putative pathogenicity islands specific for pathogenic *E. coli* strains (Moriel et al. 2010).

A CASE STUDY OF HOW VACCINE DEVELOPMENT AND UNDERSTANDING OF BACTERIAL PATHOGENESIS ARE LINKED: DISCOVERY, CHARACTERIZATION, AND EXPLOITATION OF fHbp

Reverse vaccinology, based on functionally blind selection of candidate antigens from genome screening, led to the discovery and characterization of factor H-binding protein (fHbp), which is a novel protective antigen and virulence determinant of meningococcal infection. The continuing study of fHbp has led to significant advances in the understanding of meningococcal pathogenesis, contributed to the development of improved animal models for *N. meningitidis*, and provided greater clarity for host susceptibility studies, which has in turn led to novel approaches in studying the organism itself as well as the design of more successful vaccines (Fig. 2).

Identification of fHbp as a Vaccine Antigen

fHbp was initially identified as the genome-derived *Neisseria* antigen 1870 (GNA1870), and was described as a *Neisseria*-specific putative surface lipoprotein of unknown function (Pizza et al. 2000). An unrelated vaccine discovery project also identified this antigen (referred to as lipoprotein [LP] 2086) using a proteomic approach to analyze soluble outer membrane proteins (Fletcher et al. 2004). fHbp induces high levels of bactericidal antibodies in mice (Fletcher et al. 2004; Giuliani et al. 2006) and humans (Jacobsson et al. 2009; Plested et al. 2009; Findlow et al. 2010; Giuliani et al. 2010; Halperin et al. 2010; Toneatto et al. 2011a,b). Protection against meningococcal disease correlates with the level of bactericidal antibodies in the serum bactericidal activity (SBA) assay (Borrow et al. 2006; Frasch et al. 2009), indicating that fHbp is an important vaccine antigen. Antibodies to fHbp also confer passive protection in the infant rat model of meningococcal bacteremia (Masignani et al. 2003). Development of fHbp as a vaccine antigen has greatly progressed and it is currently a component of the Novartis multivalent 4CMenB vaccine (Giuliani et al. 2006) that was submitted for licensure to the European Medicines Agency in 2010. Another vaccine contains two alleles of the recombinant fHbp protein (rLP2086) and is in late stages of development by Pfizer (Fletcher et al. 2004). In another strategy for the production of outer membrane vesicle (OMV)-based vaccines, meningococcal strains have been engineered to overexpress variants of fHbp, which has shown to provide broad protection

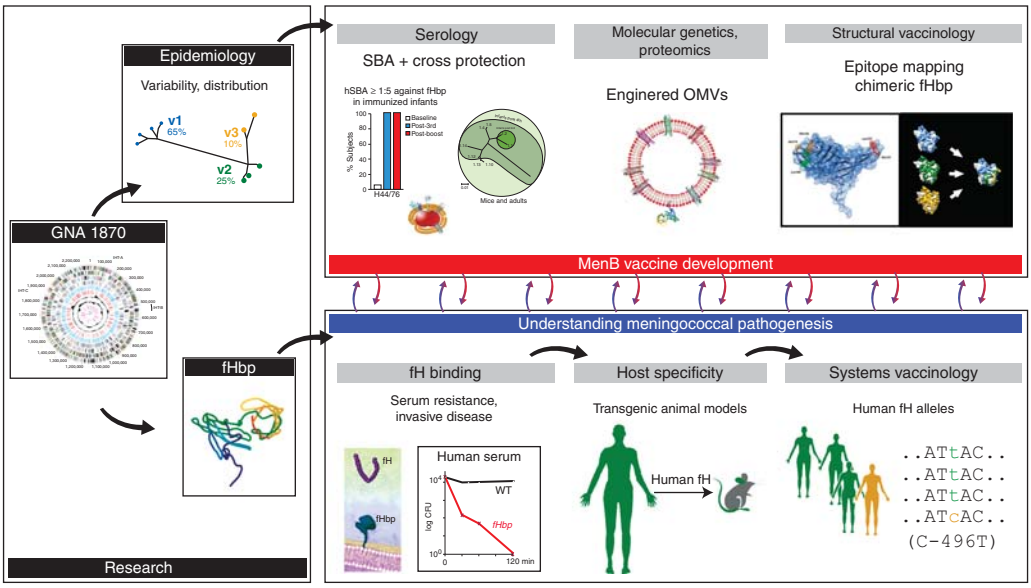

Figure 2. The meningococcal fHbp antigen as an example of how vaccine development has led to an increased understanding of pathogenesis. Genome-derived *Neisseria* antigen 1870 (GNA1870) was identified as a novel vaccine antigen from the genome sequence of *N. meningitidis* by reverse vaccinology. Since then it has been studied extensively for vaccine development (*top* panel) in terms of its distribution and sequence variation, as well as its ability to induce cross-protective bactericidal antibodies. This information has also been used to engineer meningococcal strains overexpressing fHbp for outer membrane vesicle (OMV) vaccines. In addition, structural studies have been used for epitope mapping and the generation of a chimeric fHbp antigen that is able to induce broad cross protection. Parallel to this work, the understanding of meningococcal pathogenesis has greatly advanced by studying this antigen (*bottom* panel). GNA1870 was renamed as factor H-binding protein (fHbp) owing to the discovery of its functional role in binding human factor H (fH), which increases serum resistance. This role has also led to increased understanding of meningococcal host specificity and the development of transgenic mice models expressing human fH. Furthermore, human fH alleles have been identified that increase host susceptibility to meningococcal disease.

against several MenB strains (Keiser et al. 2011; Koeberling et al. 2011a,b).

fHbp Function and Role in Pathogenesis

Before its identification as a vaccine candidate the function of fHbp was unknown. Two independent laboratories subsequently found that this antigen binds human complement factor H (fH) (Madico et al. 2006; Schneider et al. 2006), which is a key regulatory protein that acts as an inhibitor of the alternative complement pathway (reviewed in Meri et al. 2008). fHbp is an important meningococcal survival factor in human serum and blood (Madico et al. 2006; Welsch et al. 2008; Seib et al. 2009), because recruitment of fH to the bacterial surface via fHbp binding enables the pathogen to evade alternative complement-mediated killing by the host innate immune system. This has implications for fHbp not only as a virulence factor in vivo but also as a target antigen for vaccination as interestingly, anti-fHbp antibodies can also increase the susceptibility of the bacterium to killing by the alternative complement pathway by blocking fH binding to the bacteria (Madico et al. 2006). Additional functions for fHbp have also been proposed, including resistance to the antimicrobial LL-37 (Seib et al. 2009) and binding of the siderophore enterobactin (Veggi et al. 2012). Investigation of *fHbp* transcription and regulation revealed that fHbp expression is induced under oxygen limitation and implicates an important role in oxygen-poor

microenvironments, such as the submucosa or intracellular locations (Bartolini et al. 2006; Oriente et al. 2010).

Meningococcal Epidemiology and Typing

fHbp is expressed by almost all *N. meningitidis* strains studied to date, although levels of expression vary between isolates (Masignani et al. 2003; Fletcher et al. 2004; McNeil et al. 2009; Murphy et al. 2009). Furthermore, a significant degree of fHbp sequence variability exists in MenB strains circulating globally, and sequencing of *fHbp* in ~2000 MenB strains led to the identification of ~300 subvariant polypeptides (Bambini et al. 2009; Murphy et al. 2009). Different sequence variants can be classified into three groups (variants 1, 2, and 3) (Masignani et al. 2003; Brehony et al. 2009) or two subfamilies (A and B: corresponding to variants 2/3 and 1, respectively) (Fletcher et al. 2004; McNeil et al. 2009; Murphy et al. 2009; Jiang et al. 2010) that do not induce cross-protective immunity. However, all variants studied to date are able to bind fH, mediate serum resistance, and induce bactericidal antibodies (Dunphy et al. 2011; Seib et al. 2011). Members of the variant 1, 2, and 3 families are present in 65%, 25%, and 10% of the MenB global population, respectively (Beernink et al. 2007; Bambini et al. 2009; Murphy et al. 2009).

Analysis of the 4CMenB vaccine antigens fHbp, NadA, and NHBA variant distribution in meningococcal population (Bambini et al. 2009) indicated that vaccine coverage could not be predicted on the basis of multilocus sequence typing (MLST), the gold standard for meningococcal classification (Brehony et al. 2007). As a result of this, a high-throughput meningococcal antigen typing system (MATS) was developed based on a unique vaccine antigen-specific enzyme-linked immunosorbent assay (ELISA), which measures both immunologic cross reactivity and quantity of fHbp, NHBA, and NadA, and correlates with bactericidal activity (Donnelly et al. 2010). MATS analysis of large panels of strains has predicted that adults and infants immunized with the 4CMenB vaccine would be protected from disease caused by 86% and 77% of the diverse MenB strains circulating globally, respectively. This typing approach could also be applied for vaccines against other variable pathogens, including nontypable *H. influenzae*, *Streptococcus* groups A and B, and *S. pneumoniae*.

fHbp and Structural Vaccinology

Analysis of the three-dimensional structure of fHbp, by nuclear magnetic resonance (NMR) of the carboxy-terminal portion (Cantini et al. 2009) and the crystal structure of the entire fHbp protein in complex with a fragment of fH (Schneider et al. 2009) has led to identification of residues important for binding of bactericidal antibodies and fH, respectively. Using this data a structural vaccinology approach has enabled the rational design of a chimeric fHbp antigen, which combines the antigenic repertoire of the three major fHbp variant groups into a single molecule, using the variant 1 scaffold carrying patches of amino acids from the surfaces of variants 2 and 3. The chimeric fHbp protein G1 is capable of inducing antibodies that are bactericidal against a diverse panel of strains of meningococcus B, suggesting that it could be used to produce a broadly protective vaccine (Scarselli et al. 2011).

Understanding fHbp-fH Binding Leads to Better Animal Models and Improved Vaccine Candidates

Meningococcus is an obligate human pathogen that has no known reservoir outside the human host, and one of the biggest limitations in studying the pathogenesis of the disease is the lack of good animal models of infection. The finding that fHbp specifically binds human fH, but not rat or mouse fH, provided a key element to understanding meningococcal species specificity (Granoff et al. 2009). Indeed, the administration of human fH to infant rats challenged with MenB greatly increased meningococcal survival (>10-fold) (Granoff et al. 2009). Recently, a human fH transgenic mouse model has been developed by microinjection of mouse embryos with human fH cDNA, leading to concentrations of

human fH in the sera of the transgenic mice comparable to that seen in human sera (Beernink et al. 2011). This model was used to test the immunogenicity of an fHbp mutant with a single amino acid substitution, R41S, that is no longer able to bind human fH, to investigate whether epitopes may be obscured when human fH is bound to the wild-type fHbp vaccine given to humans. The fHbp R41S mutant induced higher serum bactericidal antibody responses than the wild-type fHbp and these antibodies had increased ability to block fH binding to wild-type fHbp (Beernink et al. 2011).

Host SNPs and fH Levels Are Involved in Susceptibility to Meningococcal Diseases

The increased incidence and recurrence of infection and disease in people with deficiencies (Nielsen et al. 1989) or overproduction of fH (Haralambous et al. 2006) indicated the importance of host fH in controlling meningococcal disease. A single-nucleotide polymorphism (SNP; C-496T C/C) in the promoter region of the gene encoding fH (*CFH*) is associated with slightly increased fH concentrations, reduced bactericidal activity against meningococci, and an increased risk of disease (Haralambous et al. 2006). Genome-wide association studies have also revealed a set of strongly correlated SNPs in *CFH* and the gene encoding CFH-related protein 3 (*CFHR3*) that are associated with decreased susceptibility to meningococcal disease for minor allele carriers. Interestingly, the protective alleles of the identified SNPs are rare in Africa, suggesting that these variants may play a role in the increased disease rate seen in the meningitis belt (Davila et al. 2010). However, it has been highlighted that association studies typically do not provide sufficient coverage of African populations, although new tools such as the "The 1000 Genomes Project" may help gain a better understanding of the link between host fH polymorphisms and meningococcal disease in high-risk African populations (de Bakker and Telenti 2010).

In conclusion, the discovery of fHbp through the reverse vaccinology approach and its characterization and study as a vaccine antigen has considerably advanced the understanding of meningococcal pathogenesis, and furthermore, this enhanced knowledge has led to advancements in the use of fHbp as a vaccine candidate (Fig. 2).

THE WAY AHEAD FOR VACCINOLOGY

The use of reverse vaccinology has enabled identification of numerous promising vaccine candidates against meningococcus, GBS, group A streptococcus, pneumococcus, pathogenic *E. coli*, and also for antibiotic-resistant bacteria such as *Staphylococcus aureus* (Table 1). The subsequent challenge is the refinement of these long lists of new antigens, enabling detection of the best candidate antigens and formulations to take forward for clinical development. This selection of the best candidates will largely depend on the use of novel approaches, such as structural vaccinology to engineer optimal antigens, and systems biology to gain a better understanding of signatures of immunogenicity and correlates of protection.

STRUCTURAL VACCINOLOGY

Although the mining of bacterial genome sequences has identified excellent targets that could be used in a vaccine, the sequence variability of antigens is often a major challenge to their use and development as broadly protective vaccine components. Traditionally, vaccines have been developed using natural antigens, however, rational optimization of antigens could improve their properties as immunogens by combining, exposing, and/or improving the immunogenicity of epitopes. After all, native surface proteins of pathogens have adapted to evade, not induce, immunity (Dormitzer et al. 2008).

Structural biological studies enable the atomic resolution of an antigen, and structure-based design allows the engineering of multiple immunodominant epitopes in one molecule to induce broad immune responses against different protein variants. Analogous to three musketeers, who together make a formidable team (all for one), one can combine all immunogenic

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epitopes of non-cross-reacting variants of an antigen into a rationally designed individual molecular champion (one for all), as achieved for the meningococcal fHbp (Scarselli et al. 2011) and a group B streptococcal pilus antigen (Nuccitelli et al. 2011). Structure-based antigen design has also been used in HIV vaccine development to render conserved epitopes immunogenic (Tobin et al. 2008; Burton 2010). Furthermore, stabilizing the structure of difficult antigens can improve their value as vaccine components, by optimizing their efficient production and storage stability.

SYSTEMS VACCINOLOGY

Systems biology is an interdisciplinary approach that systematically investigates the structure and complex interactions between all parts of a biological system with the ultimate goal of predicting the behavior of the system (Kitano 2002). A systems-based approach is increasingly being applied to vaccinology with two main goals in mind, first to understand the mechanisms by which vaccines stimulate protective immunity, and secondly to identify markers or molecular signatures that can be used to predict the immunogenicity or efficacy of vaccines (Pulendran et al. 2010; Nakaya et al. 2011).

Systems vaccinology, which relies on next-generation sequencing and postgenomic technologies for the high-throughput acquisition and analysis of data, can be used to define and monitor the global architecture of the human immune response and the changes that occur following vaccination. For example, analysis of vaccine-induced changes in blood, including patterns of global gene expression or alterations in immune cells, with respect to several immunological parameters, has successfully been used to identify molecular signatures induced early after vaccination with the live-attenuated yellow fever vaccine (YF-17D) that could be used to predict immunogenicity of the vaccine (Gaucher et al. 2008; Querec et al. 2009). Such signatures of immunogenicity, when identified, would also allow early identification of unsafe vaccine candidates and guide selection of the most effective vaccine composition, in terms of optimal candidate combinations, formulations, delivery systems, and immunization schemes, thus bridging the gap between antigen discovery and clinical research. Furthermore, such signatures would enable monitoring of suboptimal immune responses in certain individuals (e.g., elderly, infants, or immunocompromised populations) in whom vaccination may not confer protective immunity and may remain at risk of infection (Nakaya et al. 2011).

Other relevant data that can be gained from this field of study is the characterization of human genetic variation, at the individual or population level, and how this impacts on vaccine success. The application of state-of-the-art next-generation sequencing technology and tools that allow high-throughput detection of gene variations can provide genome-wide information, which when integrated with immune-response signature profiles will further fill the gap in our understanding of the relationship between the phenotype–genotype of an immune response. A large and growing family of polymorphisms in genes of the immune system have been identified that lead to significant variations in immune responses and that are critical to vaccine efficacy (Poland et al. 2007, 2009; Haralambieva and Poland 2010). For example, many studies have revealed associations with human leukocyte antigen (HLA) haplotypes and immune outcomes after vaccination with the MMR (measles, mumps, and rubella) vaccine (Ovsyannikova et al. 2007) hepatitis B vaccine (Kruskall et al. 1992) and the rubella vaccine (Dhiman et al. 2010). This raises the notion of "personalized vaccinology" in which a prevaccination genome-wide screen would allow individualized vaccination approaches that can be tailored and guided by the genetic uniqueness of each patient, maximizing immunogenicity and minimizing the risk of either vaccine failure or adverse events (Poland et al. 2008; Dhiman et al. 2009).

Systems vaccinology can be used to define the signatures of protection that are required to elicit a protective immune response, which will increase our knowledge of the mechanisms of action of currently successful vaccines,

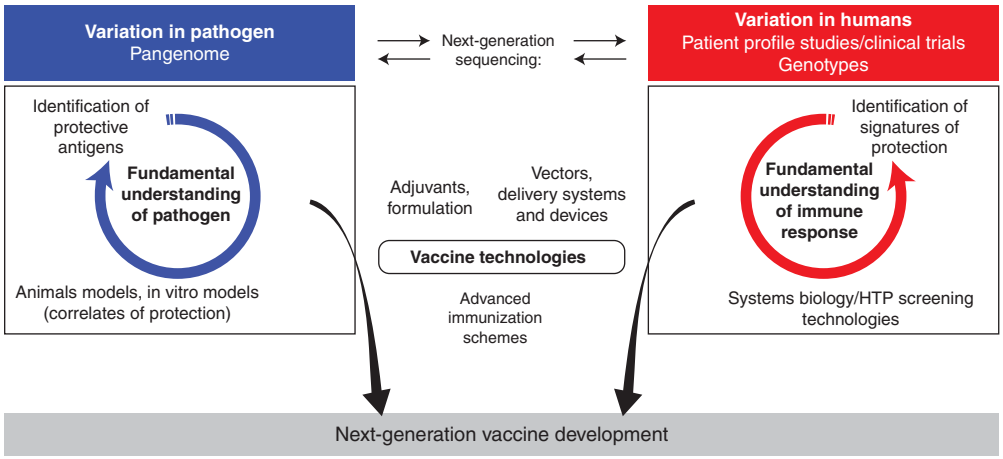

Figure 3. Key areas of next-generation vaccine development. High-throughput (HTP) DNA sequencing and screening technologies are leading to a better understanding of the genetic variation of both pathogens and the human host, which in turn is improving the fundamental understanding of the pathogen and the immune response, respectively. This information can be exploited to enable identification of protective antigens from the pathogen as well as the signatures in the host immune response that lead to protection. These two parallel fields of vaccinology, combined with improving vaccine formulation and delivery technologies, are key components of next-generation vaccine development.

as well as enable the improved identification, rational design, and testing of novel vaccine antigens (Pulendran et al. 2010; Bernstein et al. 2011).

CONCLUSIONS

With the growing problem of antimicrobial resistance, and newly emerging or reemerging pathogens (for review see Morens et al. 2008), we are increasingly looking to the development and use of vaccines to control infectious diseases. Furthermore, new and improved vaccines are needed to replace several vaccines that are suboptimal in terms of efficacy or safety. Advancing technologies continue to transform the field of vaccinology, and we are now able to use genomic-based approaches to aid selection of vaccine candidates and structure-based design to optimize the chosen immunogens. In parallel, the increasing use of systems biology will provide essential insights into the immune response elicited by vaccines, and should help identify signatures of immunogenicity and correlates of protection. This improved understanding of both the host and the pathogen is aiding the development of new vaccine technologies, including the use of small molecule adjuvants to target specific immune responses, as well as new delivery systems and immunization schemes to optimize vaccine efficacy, which are essential components for the next generation of vaccine development (Fig. 3). The knowledge gained through systems biology will also increase fundamental understanding of microbial pathogenesis, enabling continued advancements in vaccine development.

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