The Report Committee for William Joseph Kornahrens Jr.
Certifies that this is the approved version of the following report:

Recent Advances and Challenges in Antigen Engineering & Vaccine Development

APPROVED BY
SUPERVISING COMMITTEE:

Supervisor:

Jennifer Maynard

Isaac Sanchez

Recent Advances and Challenges in Antigen Engineering & Vaccine Development

by

William Joseph Kornahrens Jr., B.S.

Report

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Engineering

The University of Texas at Austin

May 2014

Dedication

I would like to dedicate this work to my wife, who has lovingly supported me throughout our time together and has helped me keep my sanity. I would also like to give a shout-out to my parents and close friends who have helped me throughout my life and continue to be an inspiration to me.

Acknowledgements

I would like to acknowledge the faculty and staff of the McKetta Department of Chemical Engineering, who have been very encouraging and supportive throughout my time at UT. I want to extend a special thanks to Dr. Jennifer Maynard for serving as my advisor and supervisor for this report.

Abstract

Recent Advances and Challenges in Antigen Engineering & Vaccine Development

William Joseph Kornahrens, Jr., M.S.E.

The University of Texas at Austin, 2014

Supervisor: Jennifer Maynard

Vaccines play a vital role in public health by preventing infectious disease across the globe. Vaccine formulations represent a weakened form of a microbe or toxin that is injected into the human body to elicit an immune response, generating antibodies to protect against a future infection. To this day, it is a challenge to identify and engineer important antigens and epitopes to focus this immune response in a safe and effective manner. The example of *Bordetella pertussis* is used to highlight the problems and lessons learned in designing a vaccine for this global epidemic. In particular, this review will focus on the advantages and disadvantages of chemical versus genetic detoxification and whole cell versus acellular vaccines in the context of pertussis. The latter part of this review will provide a summary of general strategies, such as epitope mapping and manipulation, synthesis of truncated variants, reverse vaccinology, and structural vaccinology, that have been successful in addressing increasingly complex diseases. Collectively, these techniques provide an invaluable set of tools to focus the immune response by finding and engineering specific antigens and epitopes.

Table of Contents

List of Figures

Introduction

One of the main goals of modern medicine is the prevention of infection before it occurs, especially for incurable diseases. Historically, one of the most effective ways of accomplishing this goal is through the use of vaccines. In 1796, it was Edward Jenner who introduced the concept of isolating cowpox, a naturally weakened form of smallpox in humans, and injecting it in order to induce protection.[1] Later on, Louis Pasteur conducted experiments that famously proved microorganisms were the cause of infections, and developed the "rules" by which one could prepare a vaccine: isolate, inactivate, and inject the microorganism. This strategy led to the development of many different vaccines, reducing the mortality rates for infectious diseases such as smallpox, polio, measles, and many others across the globe. Some diseases have been nearly or completely eliminated due to effectiveness of the protection and widespread use.

While it is undeniable that vaccines have had tremendous success from a public health perspective, there have been many unexpected challenges in vaccine development. It is inefficient and potentially hazardous to use the entire microorganism in a vaccine rather than specific antigens or epitopes for a targeted response that is more effective. Many issues must be addressed to identify, design, and produce immunogens so that they are safe, effective, and economical. First, this review will focus on these problems in the context of the development of pertussis vaccine, followed by short-term solutions that have been tried. The review will then conclude with an overview of general problems for vaccine development in a wide variety of diseases and discuss past and current strategies that are crucial for solving them.

Acellular Vaccines – the Pertussis Example

The very first pertussis vaccine was based on the vaccine ideas first introduced by Edward Jenner, in that the microbe *Bordetella pertussis* was chemically treated with formaldehyde and then used to make what would be known as the whole cell pertussis (wP) vaccine. However, this vaccine has been linked to inducing encephalopathy in mice, and these effects are speculated to occur in genetically susceptible patients as well.[2] These dangerous side effects and risks, though far outweighed by the benefits of wP, were part of the reason that there was a movement to develop a safer vaccine. One approach that may limit the potential for adverse reactions is to target specific, recognizable components of the microbe or its toxic products. This is useful from the practical standpoint of not having to grow cultures and process them for safety and purification. It can also be advantageous to concentrate the immune response on only the critical components needed for effective neutralization or protection.

The acellular pertussis (aP) vaccine was developed based on the idea that only specific detoxified antigens were needed to induce an immune response. The S1 subunit of pertussis toxin (PTx) was discovered to be the subunit primarily responsible for toxicity while subunits S2 through S5 were functionally used for transport and invasion of host cells. To this day, it is common to combine chemically inactivated PTx with formalin-inactivated diphtheria and tetanus toxins all into one TdaP (tetanus, diphtheria, and acellular pertussis) vaccine. Additional antigens that have been included to induce immunity to pertussis include filamentous hemagglutinin (FHA) and pertactin (PRN). Five-component vaccine formulations include the fimbrial proteins 2 and 3 (FIM2 and FIM3), which are virulence factors known to mediate binding of the bacterial cells to the lung tissue. The two vaccines currently used in the US have PTx, FHA, and PRN, either with or without FIM2 and FIM3.

Problems with Acellular Pertussis Vaccine

Unfortunately, the setbacks with the acellular pertussis vaccine provide a useful illustration of the many challenges that must be faced in vaccine development. Several studies have found indications that the DTaP vaccine does not confer long lasting immunity when compared to a previous whole cell vaccine in which the entire *Bordetella pertussis* microbe was chemically treated with formaldehyde. Ever since the acellular vaccine was introduced to the US, the number of pertussis cases has been steadily on the rise, as shown in Figure 1. In 2010, California experienced an outbreak of 9,000 pertussis cases and 10 infant deaths.[3] The Center for Disease Control and Prevention (CDC) reported that the data collected during this outbreak suggested that vaccine effectiveness was about 98% during the first year after the fifth dose of aP vaccine but declines each year, down to 71% after five years.[3] This trend is not limited to the US, as France, Italy, Japan, Finland, and other countries that recently distributed the acellular vaccine have reported a continuously increasing number of pertussis cases each year.

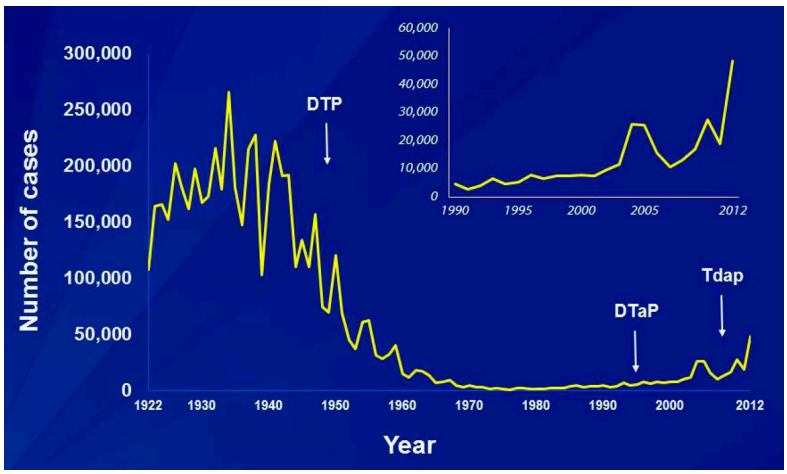

Figure 1. Reported Cases to the CDC between 1922 to 2012.[3] DTP is another name for whole cell vaccine, DTaP is the acellular vaccine, and Tdap is the acellular vaccine with a reduced level of diphtheria toxoid.

Mutations to Evade Neutralization

In 2012, there were 42,000 cases of pertussis in the US, making pertussis the most common vaccine-preventable illness in the country. There has been much speculation on

possible hypotheses to explain why pertussis is reemerging. One possibility is that *B. pertussis* may be evolving and slowly adapting to the challenge and selection set forth by the acellular vaccine. It is possible that mutations in the genes for the antigens contained in aP may be a way for *B. pertussis* to adapt and survive in humans who have received the vaccine. There is evidence that this has already occurred in France and Finland for the antigen PRN, which is thought to be an important adhesion used by the bacterium for attaching to epithelial cells.[4] After aP was administered in these countries, *B. pertussis* strains lacking the expression of PRN became more prevalent, and studies examining macrophage cytotoxicity and the murine model of infection have shown these strains to be just as lethal as the strains that do express PRN.[5] One study found that strains expressing a combination of the *prn2-ptxP3* alleles were the most prevalent during an epidemic in Australia. This was explained by previous evidence indicating that the *prn2* allele had been shown to confer an advantage to selective pressure from the acellular vaccine while the *ptxP3* allele was associated with higher hospitalization and mortality rates due to increased production of pertussis toxin.[6] An additional study that examined changes in pertussis strains during a 2008-2010 Japanese pertussis epidemic found that strains expressing the fim3B mutation increased dramatically during this time frame while strains that did not express PRN were decreased by half.[7] These facts indicate that strains expressing the fim3B mutation may have been the more likely cause of this particular epidemic in Japan.

Memory T-cell Responses

Another factor that could possibly contribute to the recent uptick in pertussis cases could be the skewing of the immune response in children. While aP is very effective at eliciting a strong response to the antigens contained in the vaccine, this may not be as effective in the long run as the whole cell vaccine's induction of a broad adaptive response to many antigens simultaneously. Adaptive immunity is determined in large part by Th1 and Th2 responses. CD4+ T-cell helpers are important in mediating and coordinating the immune response. Several studies have shown that infant immunization with aP results in a skewed Th2 response and relatively weak Th1 response, which puts

the children at a long-term disadvantage with regard to cellular memory immune responses to pertussis.[8] This contrasts with the wP vaccine which elicits a generally weaker response to a larger variety of antigens.

Recent evidence has shown that Th17 is important in recruiting neutrophils at mucosal surfaces to control extracellular bacterial infections. Warfel et al. studied the responses of infant baboons immunized with aP, wP, or neither at 2, 4, and 6 months and challenged with pertussis at 7 months.[9] One unique aspect of this study is that the common method of transmission in humans by coughing is preserved in this animal model of disease. They found that, while aP and wP induced high levels of response to the antigen components and pertussis symptoms were reduced, this did not prevent transmission and colonization of the disease in naïve baboons, as measured by nasopharyngeal washes. A separate part of the study measured cytokine responses in fractions of peripheral blood mononucleated cells incubated ex vivo with heat-treated *B. pertussis* in order to identify the associated memory T-cell responses. They found that the T-cell responses were different in that aP resulted in Th1/Th2 while wP induced Th17 and Th1 memory responses, which more closely matched the responses by individuals with previous natural infection. They concluded that this difference in response was an indication that the aP response is mismatched to the disease and that more research needs to be done to determine how Th17/Th1 responses are stimulated and more effort should be made to produce vaccines that match these responses.

Chemical and Genetic Inactivation

The initial method for detoxifying antigens generally involved incubating them in chemicals such as formaldehyde, glutaraldehyde, hydrogen peroxide, and various other chemicals. Like many of the best discoveries, this method was developed by complete accident when Gaston Ramon had decided to store the culture supernatant of *Corynebacterium diphtheriae* in formaldehyde to help prevent contamination.[10] He later discovered that prolonged storage in formaldehyde resulted in the loss of the toxin's ability to kill animals and conferred resistance to future injections of the toxin. Formaldehyde is very popular and has been used in the chemical detoxification of tetanus

and pertussis toxins for vaccine development. Unfortunately, chemical detoxification is not always ideal since it has been shown to cause reduced immunogenicity and residual toxin activity as a result of changes in the antigen's conformation. In addition, the chemical detoxification could lead to large batch variability and expensive testing to ensure that the toxoid would not revert back to a potent toxin.

The main concept behind genetic inactivation of toxins is to make mutations and then select for the mutant versions that offer the best immunogenicity and lowest toxicity. For example, the National Institute of Allergy and Infectious Diseases (NIAID) produced genetically inactivated forms of PTx.[11] Later, clinical trials by Podda et al. and others found that the 9K/129G PTx mutant (Arg-9→Lys and Glu-129→Gly) was both safe and immunogenic in children and infants, making it an ideal candidate for a new vaccine.[12] Furthermore, NIAID studies showed that vaccines with 5-10 ug of PTx mutant produced the same or higher levels of IgG anti-PT as 25 ug of chemically detoxified PTx, which suggests that the mutant PTx is five times as immunogenic and would confer immunity for a longer period of time.[13] Despite the potential benefits of using the new genetically detoxified antigens, patents and excessive costs for testing have deterred vaccine manufacturers from pursuing the production of the genetically modified version of PTx.

Advances in Vaccine Development

Regardless of why the acellular vaccine confers only short-term immunity, there is a clear need to improve upon this vaccine. There is somewhat of a disadvantage in the fact that research has not been focused on pertussis for a long time since the problem was long thought to be solved effectively by the acellular vaccine. However, there have been several new methods and advances in vaccine development and antigen design since acellular vaccine was first distributed in the early 1990's. These methods will be discussed in their previous application to other diseases for which vaccine formulation is not straightforward.

Epitope Mapping

Sometimes it is desirable to work backwards from a known highly potent antibody to determine the corresponding epitope, or where on the antigen it will bind. Similar to the acellular or subunit approach discussed earlier, it makes intuitive sense that reducing vaccine formulations to contain only the desired epitope would focus the immune response even further on the crucial parts of the antigen that are most effective at neutralization, speeding up clearance of the disease. Epitopes can either be linear sequences of amino acids modeled as a single peptide or discontinuous conformational parts of the antigen that are brought closely together in the native state.

Given the critical importance of epitopes, it is not surprising that there are a variety of methods that have been developed in order to find them and characterize their interactions with antibodies. One of the first ways used to do this was via "pepscans," an assay introduced by Mario Geysen in 1984 that essentially screened for simple linear epitope sequences using an array of peptides assessed by binding. The binding of these peptides would then reveal the important sequence of this epitope and could be tested for immunogenicity.[14]

The problem is that many important epitopes depend on the antigen's conformation and folded structure, not just a linear sequence of amino acids. The two main biophysical methods used for these epitopes would be X-ray crystallography and nuclear magnetic resonance (NMR). Crystallization is by far the most rigorous assay,

utilizing x-ray diffraction of the crystal structure of an antibody bound to the antigen. Using the data acquired, the electron densities of the amino acids in a 3D structure can be precisely mapped, indicating where the interface is between the antibody and antigen.[15] The major hurdle with this method is that it is very complicated in execution compared to other methods and the success rate is fairly low, in part because it is a challenge to crystallize the antibody and antigen. In contrast, NMR is used to analyze solutions containing these complexes by measuring the changes in electromagnetic radiation absorption and emission under an applied magnetic field.[15] It has similar disadavantages to X-ray crystallography, and it is limited to the analysis of proteins 30 kDa or smaller. Both of these methods are good at defining the interface between antigen and antibody, but there may be some disagreement between the two assays about which residues play an important role at the interface. For the cases where it is difficult to analyze the antigen-antibody complex, one can use crystal structures of the individual antigen and corresponding antibody to examine computationally how they fit together in a computer. However, there are also many limitations to this since the target antigen should be rigid (which is characteristic of small molecules), and even then the predicted interface may not closely match what happens in nature.

Another set of assays used to determine the important epitopes can be categorized as functional binding assays, which would include ELISAs, dot blots, and Western blots. The idea is to use certain parts of the antigen to test for binding so that the important epitopes are found by antibody binding to smaller and smaller fragments. One of the ways in which this is done is through site-directed mutagenesis, where amino acid residues are altered randomly or systematically, and the effects on binding are measured. Systematic mutations can be done via alanine scanning, where residues are replaced with alanine one at a time.[16] This method can be a time-consuming task due to the sheer number of mutants that must be synthesized, purified, and characterized for comparison. One of the interesting insights that came from this assay was that the manipulation of residues outside of the interfacial contact area between antigens and antibodies could have more of an impact on binding than those at the interface. The concept that

preserving overall epitope structure may sometimes be more important than the residues in direct contact with the antibody forms the basis behind structural vaccinology, a novel method that is discussed later in this report.

The other major method of searching for these epitopes is by using a combinatorial approach with phage display peptide libraries. Large numbers of short random peptides can be displayed on the phage surface and screened for affinity to an immobilized antibody. The DNA contained within the phages that bind can then be sequenced to determine the epitope.[15] If this technique is used repeatedly until the phage that binds most effectively is left, then it can be used as a possible candidate for vaccines. Alternatively, it could be attempted to use computational algorithms to find where the peptides that bind could correspond to the epitope.

T-cell Epitopes

Unlike B-cell epitopes, T-cell epitopes are typically linear and not dependent on conformational structure. They are presented on the surface of antigen presenting cells (APCs), stimulating an immune response when bound by major histocompatibility (MHC) molecules. Since T-cell epitopes are relatively easier to work with, several methods have been developed to characterize and modify them.

One way in which T-cell epitopes have been employed is by increasing the immune response. In one example involving an anthrax vaccine candidate, Oscherwitz et al. had previously found that a specific B-cell epitope (the loop-neutralizing determinant, or LND) located on the 2B2-2B3 loop of protective antigen stimulated high immune responses.[17] However, some of the rabbit models showed no response at all, and it was hypothesized that this was due to the lack of a T-cell epitope contained in the sequence, limiting the ability of MHC-II to recognize the antigen. However, when the LND was synthesized with the P30 T-cell helper epitope of tetanus toxin, this variant consistently neutralized lethal toxin in mice at a higher antibody titer and higher affinity than LND without this epitope.

It may also be desired to decrease the immunogenicity of certain proteins while not affecting functionality. This can involve dramatic changes in the T-cell epitopes that

may not be possible to discover with conventional methods. In one case, the enzyme used to treat childhood acute lymphoblastic leukemia, *Escherichia coli* L-asparaginase II (EcAII), is highly immunogenic, a problem that plagues many heterologous enzymes that could be used to treat cancer and other diseases. Cantor et al. were successful in using a computational approach to identify T-cell epitopes to test by randomizing the important residues with saturation mutagenesis followed by a neutral drift screen so that the enzyme changed under selective conditions but did not lose functionality.[18] Finally, immunogenicity and epitope recognition are tested by antibody titer and T-cell activation assays.

Multiple Epitopes and Truncated Variants

Another way that vaccine development is complicated with regard to epitopes occurs when different strains of the same microbial species have slightly different epitopes for the same antigen or have multiple antigens/toxic products that must be targeted for an appropriate immune response, making it difficult to produce a single vaccine that would be effective at providing protection on a global scale. This problem of having multiple epitopes for the same antigen is particularly relevant to lyme disease, which is caused by bacteria (belonging to the *Borrelia* genus) carried in ticks. The outer surface protein A (OspA) has been successfully used as an antigen in vaccines for lyme disease in the US, where only *Borrelia burgdorferi* is prevalent. However, this protein has many distinct epitopes that vary across the many genetically diverse *Borrelia* species that are prevalent in Europe and Asia. Current research efforts have attempted to circumvent this problem entirely by making three recombinant OspA proteins, each of which contains protective epitopes for two distinct serotypes. These chimeric molecules are fusions of the proximal and distal portions of the OspA sequences that preserve the native three-dimensional structure, and they were expressed in *Escherichia coli* with a T7 expression system.[19] An example of one of these recombinant antigens is shown below in Figure 2. Experiments in mice and human clinical trials demonstrate great potential for vaccines with these three antigens to provide universal protection across the six different serotypes.

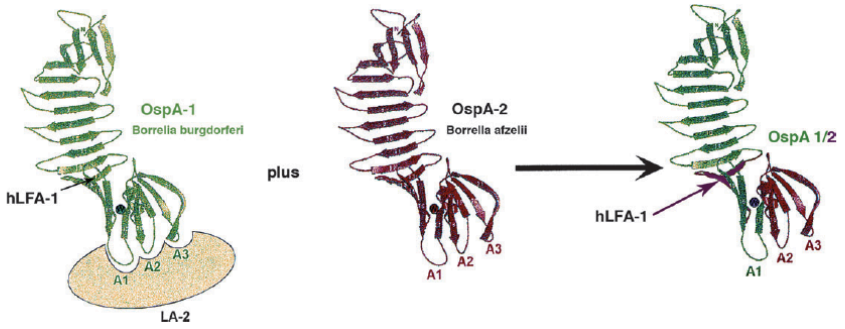

Figure 2 – Recombinant OspA.[19]

Decoy Epitopes

While using immunogenic, nontoxic antigens as vaccine components is an appealing strategy, it is important to note that this approach is only effective if the functionally important parts or epitopes of the antigens are recognized by the immune system. The problem is that it is not always easy to choose the right epitope, and the immune system may preferentially recognize a different epitope based on accessibility and abundance. This issue is what makes vaccine development so difficult, particularly in the case of viruses that use decoy epitopes on structural proteins that effectively "hide" the toxic protein and/or viral DNA until it has already invaded the cell.

For example, HIV utilizes highly variable regions on the outer envelope protein that are immunodominant but do not ultimately impair the ability of the viral DNA to invade and replicate inside host cells. Likewise, influenza virus uses surface hemagglutinin (HA) to bind and enter cells, but epitopes near HA's receptor binding site are highly variable. This problem is further exacerbated by antigenic drift, which are mutations that accumulate slowly over time, and antigenic shift, which is when viruses affecting the same host can exchange segments of RNA in an event similar to crossing over that results in mutant versions of HA for which humans have no pre-existing immunity.

Reverse Vaccinology

Reverse vaccinology, a concept developed in 2000 by Rino Rappuoli, is the process of using bioinformatics to screen the entire pathogenic genome for suitable vaccine candidates. The traditional process of growing up bacterial cultures, isolating antigens, and testing them with biochemical assays and in animal models can be costly, time consuming, and is limited by only focusing on proteins that are highly immunogenic but do not necessarily result in protective immunity for the reasons discussed previously. Reverse vaccinology counteracts these issues by being able to look at virtually every possible antigen contained in the bacterial genome, avoiding the inherent bias of immunogenicity or ability to generate large quantities of the antigen in a laboratory setting.

One of the very first examples of success using this method was in the development of a new vaccine for serotype B *Neisseria meningitidis,* which can cause meningitis by crossing the blood-brain barrier, evading the immune system in a polysaccharide capsule. For most serotypes (A, C, Y, and W135), vaccines designed to enlist bactericidal antibodies to attack the capsule are effective, but the capsule for serotype B is a special case since it is identical in composition to the polysialic acid present in human glycoproteins, eluding an immune response as a human self-antigen and making it difficult to apply traditional techniques to find protective epitopes. Reverse vaccinology came into play by using bioinformatics analysis of the entire bacterial genome to predict which of the total 2158 proteins were exposed on the surface or secreted to the extracellular milieu.[20] About 350 of these antigens were then expressed and purified from *E. coli* cultures and tested in mice. Serum samples from these mice were then be tested for protection via a bactericidal assay, leading to five previously undiscovered vaccine candidates that were combined in a formulation known as 4CMenB, which just completed clinical trials.[21]

What if there are several bacterial strains with no viable universal vaccine candidate? Such was the case with Group B *streptococcus*. Using reverse vaccinology by sequencing eight bacterial genomes representative of this strain, it was found that about

589 genes were predicted to be proteins expressed on the surface, and ultimately four of them showed evidence of protection in an animal model.[22] However, none of the four antigens would have been detected through the use of traditional methods to find a universal candidate for all of the strains; three of them were not expressed in all eight genomes, and the one protein that was expressed in all of the genomes was not exposed on the surface for all of the strains. This scenario in particular highlights the need to screen multiple genomes that not only express antigens of interest but also take into account the variation in surface availability and expression levels.

Structural Vaccinology

Another new approach that is starting to show promise in vaccine development is structural vaccinology. Rather than focusing on the sequences of important epitopes, this method places more importance on preserving the core structure of the antigen so that the folding of the overall epitope is preserved. This can also be used in conjunction with reverse vaccinology once suitable epitopes have been found, as depicted in Figure 3 below. There are several cases already for which this method has been used successfully.

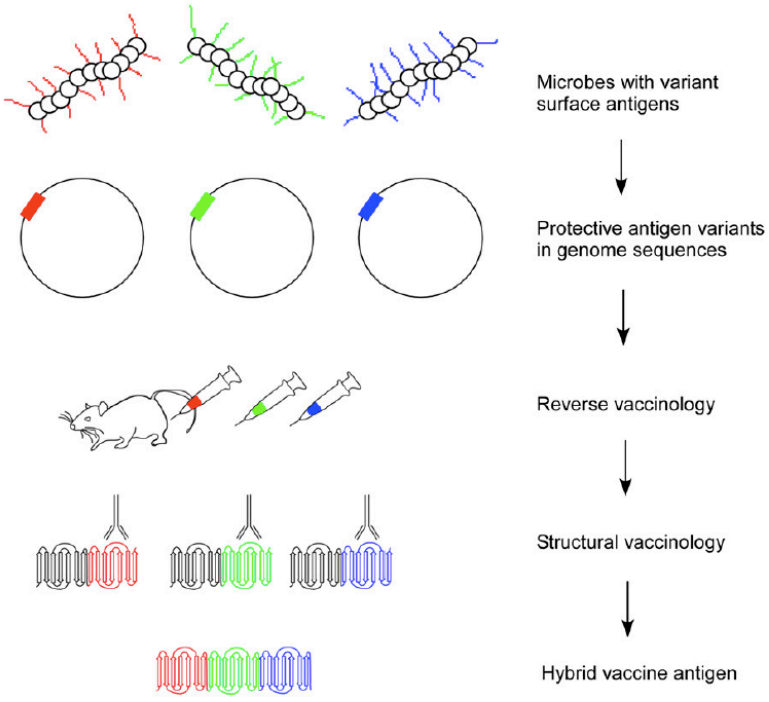

Figure 3 – Reverse Vaccinology and Structural Vaccinology.[23]

As described before, the surface-exposed factor H-binding protein (fHbp) has been identified by reverse vaccinology as a highly immunogenic antigen, but there are over 500 different variants that can be classified into three main groups that do not elicit cross-protection for each other. This implies that any effective vaccine would require at least three antigens in the formulation, making production complicated and expensive when compared to the ideal case of a single antigen. The first attempt to make a single universal antigen was to insert amino acid sequences from the other variants into the gene for variant 1, but the failure of this approach suggested that the 3D structures of each epitope would also need to be preserved for protection. By dividing the 1 C terminus into separate patches large enough (900 to 2000 A) to preserve epitope structure while inserting key amino acid sequences where they would be most exposed to the solvent, an antigen was made that generated antibodies to all three variants.[24]

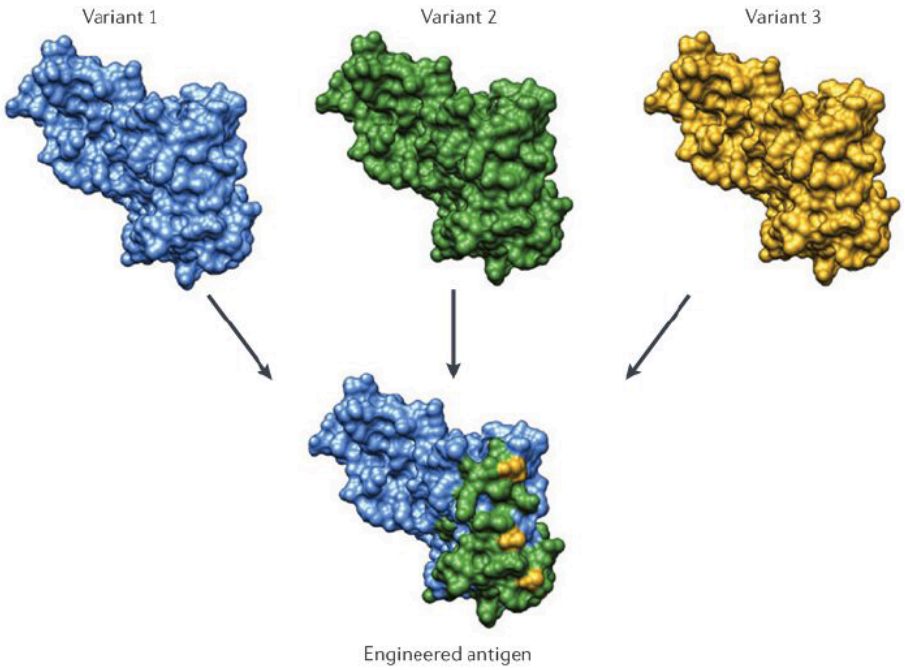

Figure 4 – Structural Vaccinology Strategy with fHbp.[24]

In other cases, this approach has shown that antigens mirroring specific structural domains of proteins may be all that is required to elicit an effective immune response that is broadly protective, as was the case for the BP-2a backbone protein variant of Group B *streptococcus* (GBS).[25] Due to the reverse vaccinology work discussed previously, it was revealed that all GBS strains express pili, structures composed of a backbone protein and two ancillary proteins. The backbone component has been shown to be protective, but its selection as an antigen is complicated by the eight different variants that are possible. However, the 3D structure for the six variants corresponding to one of the three genomic pilus islands (BP-2a) showed that the backbone was organized into four domains (D1 through D4), of which D3 was primarily exposed for binding and triggered opsonophagocytotic antibodies. It was later shown that the D3 domains of each variant, when combined into one recombinant antigen, were enough to induce protection from all six variants of BP-2a.[25]

Another important example is in the formulation of a vaccine for respiratory syncytial virus (RSV) that is now in the clinical trial phase. Some structural studies have shown that targeting the fusion (F) glycoprotein would be ideal. However, the problem is that this protein undergoes changes in conformation (from a pre-fusion form to a transient intermediate to a post-fusion form) during invasion of host cells. Since protection is mainly hypothesized to come from the prevention of invasion into host cells, it makes intuitive sense that the initial conformation of the F glycoprotein would be the desired one to target. Unfortunately, this conformation is difficult to isolate since extraction by detergent and other methods makes it quickly shift to the post-fusion form. Using previous experience with the engineering of the HPIV-3 post-fusion protein, researchers modified the post-fusion F glycoprotein of RSV by manipulating the fusion protein region to simultaneously reduce hydrophobicity, increase thermostability, and increase homogeneity.[26] These properties are highly desired for antigens since they must be hydrophilic for purification, stable enough for a long shelf life, and able to crystallize for ease of characterization. It was unexpected for this antigen to be as efficient at neutralization since it was expected that the epitopes on the antigen surface would be

folded differently and buried inside the pre-fusion conformer. The crystal structure of the engineered F glycoprotein revealed that an alpha helix insertion had caused the protective epitopes to be exposed, and that these epitopes were largely unaffected by the switch between the pre-fusion and post-fusion structures.[24]

Conclusion

Given the information presented here, it is clear that there are many factors to take into consideration when selecting and designing antigens for a particular pathogen. For the most challenging infectious diseases, it is no longer sufficient to blindly screen for antigens that are the most immunogenic. In order to generate an appropriate immune response, future antigens must be assessed by strategically screening the genomic information for potential protective antigens, examining the epitopes that are available, and preserving the crucial structures required for immunogenicity.

There are many new promising approaches that take advantage of the recent strides made in high throughput screening and genomic research. Reverse and structural vaccinology have already been shown to be successful at making progress in discovering suitable vaccine candidates that may have been impossible to find or, at the very least, difficult and time consuming to search for using traditional techniques. It will become increasingly crucial to determine how to leverage the vast amount of genomic information from multiple strains to formulate universal vaccines with antigens that stimulate broad protection in disease variants on a global scale, making them more economical and effective.

References

1. Jenner, E. (1801). *The Origin of the Vaccines Inoculation* (London: Shury).

2. Steinman, L., Weiss, A., Adelman, N., Lim, M., Zunega, R., Oehlert, J., et al. (December 1985). Pertussis toxin is required for pertussis vaccine encephalopathy. *Proceedings of the National Academy of Sciences*, *82*, 8733-8736.

3. Edwards, K. (January 2014). Unraveling the challenges of pertussis. *Proceedings of the National Academy of Sciences*, *111*, 575-576.

4. He, Q. (August 2012). Appearance of *Bordetella pertussis* strains not expressing the vaccine antigen pertactin in Finland. *Clinical and Vaccine Immunology*, *19*, 1703-1704.

5. Hergerle, N., Paris, A., Brun, D., Dore, G., Njamkepo, E., Guillot, S., et al. (May 2012). Evolution of French *Bordetella pertussis* and *Bordetella parapertussis* isolates: increase of Bordetellae not expressing pertactin. *Clinical Microbiology and Infection*, *18*, E340-E346.

6. Octavia, S., Sintchenko, V., Gilbert, G., Lawrence, A., Keil, A., Hogg, G., et al. (March 2012). Newly emerging clones of *Bordetella pertussis* carrying prn2 and ptxP3 alleles implicated in Australian pertussis epidemic in 2008-2010. *Journal of Infectious Diseases*, *205*, 1220-1224.

7. Miyaji, Y., Otsuka, N., Toyoizumi-Ajisaka, H., Shibayama, K., & Kamachi, K. (October 2013). Genetic analysis of *Bordetella pertussis* isolates from the 2008–2010 pertussis epidemic in Japan. *PLOS One*, *8*, 1-8.

8. Poland, G. (September 2012). Pertussis outbreaks and pertussis vaccines: New insights, new concerns, new recommendations? *Vaccine*, *30*, 6957-6959.

9. Warfel, J., Zimmerman, L., & Merkel, T. (January 2014). Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proceedings of the National Academy of Sciences*, *111*, 787-792.

10. Rappuoli, R. (1994). Toxin inactivation and antigen stabilization: two different uses of formaldehyde. *Vaccine*, *12*, 579-581.

11. Burnette, W., Ciepalk, W., Mar, V., Kaljot, K., Sato, H., & Keith, J. (October 1988). Pertussis toxin S1 mutant with reduced enzyme activity and a conserved protective epitope. *Science*, *242*, 72-74.

12. Podda, A., De Luca, E. C., Titone, L., Casadei, A., Cascio, A., Peppoioni, S., et al. (December 1991). Acellular pertussis vaccine composed of genetically inactivated pertussis toxin: Safety and immunogenicity in 12- to 24- and 2- to 4-month-old children. *The Journal of Pediatrics*, *120*, 680-685.

13. Robbins, J., Schneerson, R., Keith, J., Shiloach, J., Miller, M., & Trollors, B. (January 2007). The rise in pertussis cases urges replacement of chemically-inactivated with genetically-inactivated toxoid for DTP. *Vaccine*, *25*, 2811-2816.

14. Regenmortel, M. V., & Pellequer, J. (1994). Predicting antigenic determinants in proteins: looking for unidimensional solutions to a three-dimensional problem? *Peptide Research*, 7, 224-228.

15. Gershoni, J., Roitburd-Berman, A., Siman-Tov, D., Freund, N., & Weiss, Y. (2007). Epitope mapping: The first step in developing epitope-based vaccines. *Drug Development, 21*, 145-156.

16. Cunnignham, B., & Wells, J. (January 1989). High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science*, *244*, 1081-1085.

17. Oscherwitz, J., Yu, F., & Cease, K. (December 2009). A heterologous helper T-Cell epitope enhances the immunogenicity of a multiple-antigenic-peptide vaccine targeting the cryptic loop-neutralizing determinant of *Bacillus anthracis* protective antigen. *Infection and immunity, 77*, 5509-5518.

18. Cantor, J., Yoo, T. H., Dixit, A., B., I., Forsthuber, T., & Georgiou, G. (January 2011). Therapeutic enzyme deimmunization by combinatorial T-cell epitope removal using neutral drift. *Proceedings of the National Academy of Sciences*, *108*, 1272-1277.

19. Livey, I., O'Rourke, M., Traweger, A., Savidis-Dacho, H., Crowe, B., & Barrett, P. (2011). A new approach to a lyme disease vaccine. *Clinical Infectious Diseases*, *52*, s266-s270.

20. Donati, C., & Rappuoli, R. (2013). Reverse vaccinology in the 21st century: improvements over the original design. *Annals of the New York Academy of Sciences*, *1285*, 115-132.

21. Giuliani, M. M., Adu-Bobie, J., Comanducci, M., Arico, B., Savino, S., Santini, L., et al. (July 2006). Inaugural article: A universal vaccine for serogroup B meningococcus. *Proceedings of the National Academy of Sciences*, *103*, 10834-10839.

22. Maione, D., Margarit, I., Rinaudo, C., Masignani, V., Mora, M., Scarselli, M., et al. (July 2005). Identification of a universal Group B Streptococcus vaccine by multiple genome screen. *Science*, *309*, 148-150.

23. Schneewind, O., & Missiakas, D. (June 2011). Structural vaccinology to thwart antigenic variation in microbial pathogens. *Proceedings of the National Academy of Sciences*, *108*, 10029-10030.

24. Dormitzer, P., Grandi, G., & Rappuoli, R. (December 2012). Structural vaccinology starts to deliver. *Nature Reviews Microbiology*, *10*, 807-813.

25. Nuccitelli, A., Cozzi, R., Gourlay, L., Donnarumma, D., Necchi, F., Norais, N., et al. (June 2011). From the Cover: Structure-based approach to rationally design a chimeric protein for an effective vaccine against Group B Streptococcus infections. *Proceedings of the National Academy of Sciences*, *108*, 10278-10283.

26. Swanson, K., Settembre, E., Shaw, C., Dey, A., Rappuoli, R., Mandl, C., et al. (June 2011). Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. *Proceedings of the National Academy of Sciences*, *108*, 9619-9624.