CHAPTER 3

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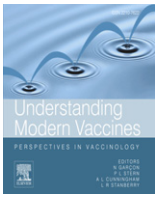
Understanding Modern Vaccines:
Perspectives in Vaccinology



Vaccine antigens

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Key concepts

- Many vaccines are comprised of whole viruses or bacteria and therefore contain many, often poorly defined, antigens as well as other microbial molecules important in triggering innate and/or adaptive immune responses
- Where the whole pathogen approach is not feasible or desirable, other approaches are considered, such as subunit antigens that are naturally derived or generated using recombinant DNA technology
- Vaccines containing fewer defined antigens may be less reactogenic but also less immunogenic thus necessitating the inclusion of adjuvants
- Key pathogen virulence determinants usually make excellent antigens for inclusion in vaccines, eg viral ligands such as haemagglutinins or inactivated bacterial toxins
- The final choice of antigen is often determined by what is achievable immunologically and technologically, and what is optimal from a safety perspective
- An immunogen is an antigen capable of inducing an adaptive immune response; an epitope is the highly specific structure or site on an antigen that is recognised by either the surface B-cell receptor, T-cell receptor or soluble antibody

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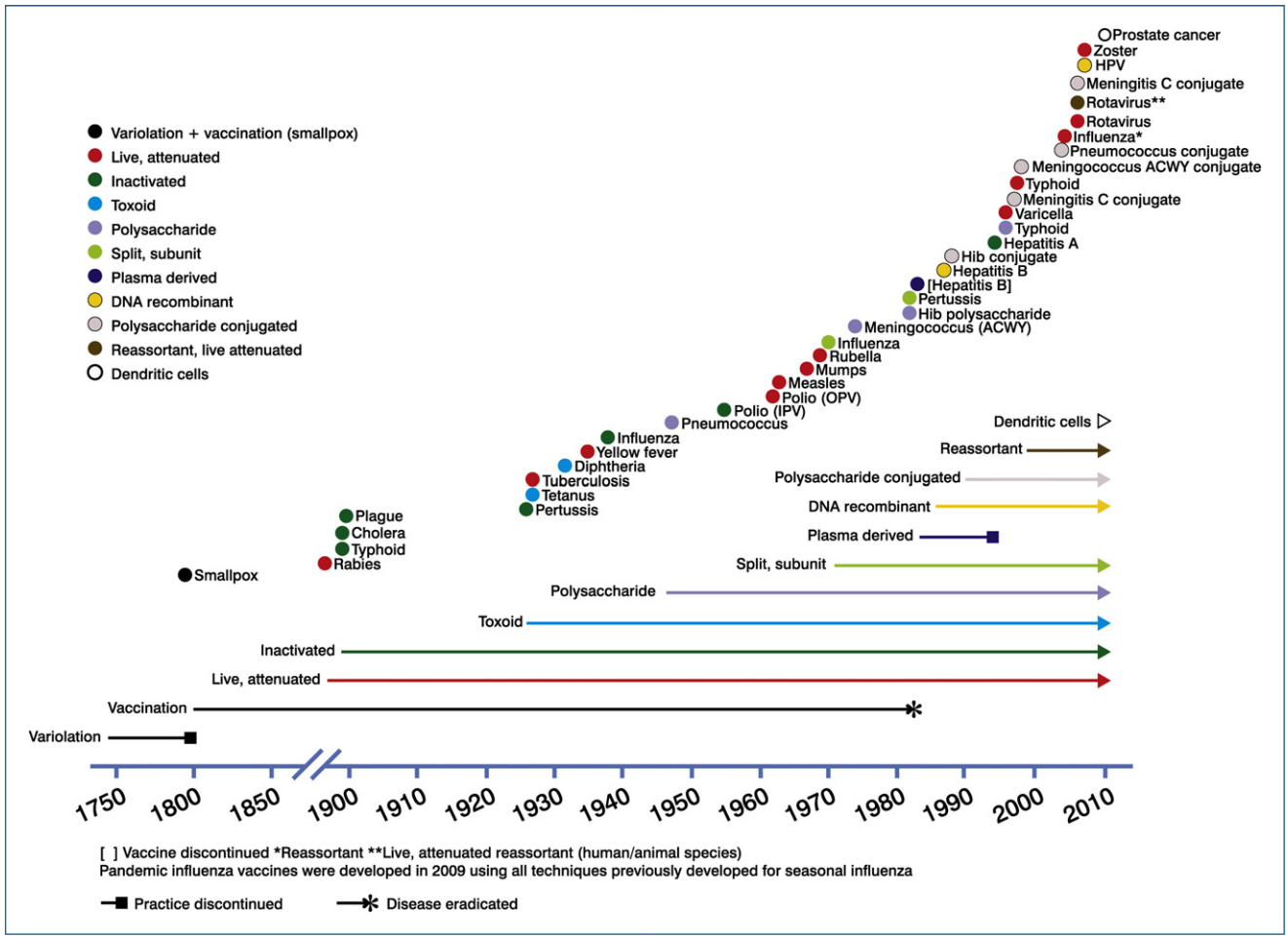
Vaccine *antigens* include whole live pathogens (modified to reduce their *virulence*), individual pathogen components (eg protein or polysaccharides) and the genetic material of the pathogen (ie 'naked' DNA/RNA) which can direct the production of the vaccine antigen in the recipient.

The earliest vaccine consisted of infected fluid derived from people infected with cowpox, which was used by Edward Jenner to prevent the significantly more serious human disease of smallpox. What Jenner did not know was that the infected fluid used contained live cowpox virus. Cowpox virus shares antigenic components with smallpox, but is much less virulent or pathogenic in humans. Consequently, vaccinees developed immunity to smallpox without the risk of serious disease. Subsequent empirical observations in the 19th century noted that pathogens with reduced virulence and even dead pathogenic bacteria also acted as vaccines. This breakthrough allowed the development of attenuated and inactivated whole-pathogen vaccines, pioneered by the work of Louis Pasteur and Robert Koch. A paradigm shift occurred in the late 19th and early 20th centuries as a result of progress in biochemistry and the development of vaccines based on toxins, or their inactivated derivatives, the *toxoids* (Figure 3.1). The realisation that the whole pathogen was not always needed to induce immunity, and the subsequent concept of 'antigen', were essential to improvements in the safety and efficacy of *prophylactic vaccines*. It is important to note that most vaccines in this period were successfully developed in the absence of a solid understanding of the immunological responses induced by vaccines or key physical structures of the targeted pathogens. Today, a better understanding of host–pathogen interactions and of the key features needed to induce a proper immune response allows for a more scientific (rational, hypothesis-based approach), rather than empirical (trial and error), approach to the choice and definition of the target antigen(s).

Figure 3.1 Vaccines and technologies. Vaccine development timeline from the first practice of variolation – deliberate infection of humans with material derived from human smallpox pustular material. Most of the other technologies are still used for the development of vaccines. Plasma-derived vaccines have not been used in most countries since the 1990s.

HPV, human papillomavirus; Hib, Haemophilus influenzae type b; OPV, oral polio vaccine; IPV, inactivated polio vaccine.



Antigen discovery and definition

In the late 19th and early 20th centuries, bacterial constituents were defined as 'antigen', and later as '*immunogen*'. Paul Ehrlich formulated the theory that physiologically active substances interacted with specific receptors in the blood and introduced the concept of antigens ('**anti**body **gen**erators') as the ligands of *antibodies*. This idea was developed from his work on toxin–*antitoxin* complexes in sera, and the first recognition of antibodies in 1890. An antigen may be defined as the target of an immune response – this may be an innate or adaptive response. How the immune system receives the information around the antigen is extremely important as well. The receptors of *T* and *B cells* specifically recognise limited and unique parts of an antigen molecule during an adaptive immune response; therefore the selection of the appropriate antigen is central to vaccine design. In addition to these specific antigenic components, there are several other types of pathogen constituents that are essential to the induction of innate and subsequent adaptive immune responses, which may be considered as 'defensive triggers'. These are needed together with the antigenic structure to activate the immune response (see *Chapter 2 – Vaccine immunology*).

Identifying and producing vaccine antigens

The identification of vaccine antigens can vary in complexity depending on whether the whole pathogen or pathogen-derived material is involved (Figure 3.2). Pathogen-based approaches to vaccine antigens can vary in terms of the complexity of the material they contain. This may include the use of whole viruses or bacteria, in the form of *reassortant*, attenuated or inactivated *microbes*. Attenuated pathogens remain live and replication-competent but are altered in some way to reduce their virulence in the target host; inactivated pathogens are dead, or in the case of viruses inactivated, eg unable to replicate; reassortant pathogens are a subtype of attenuated organisms, containing genetic material

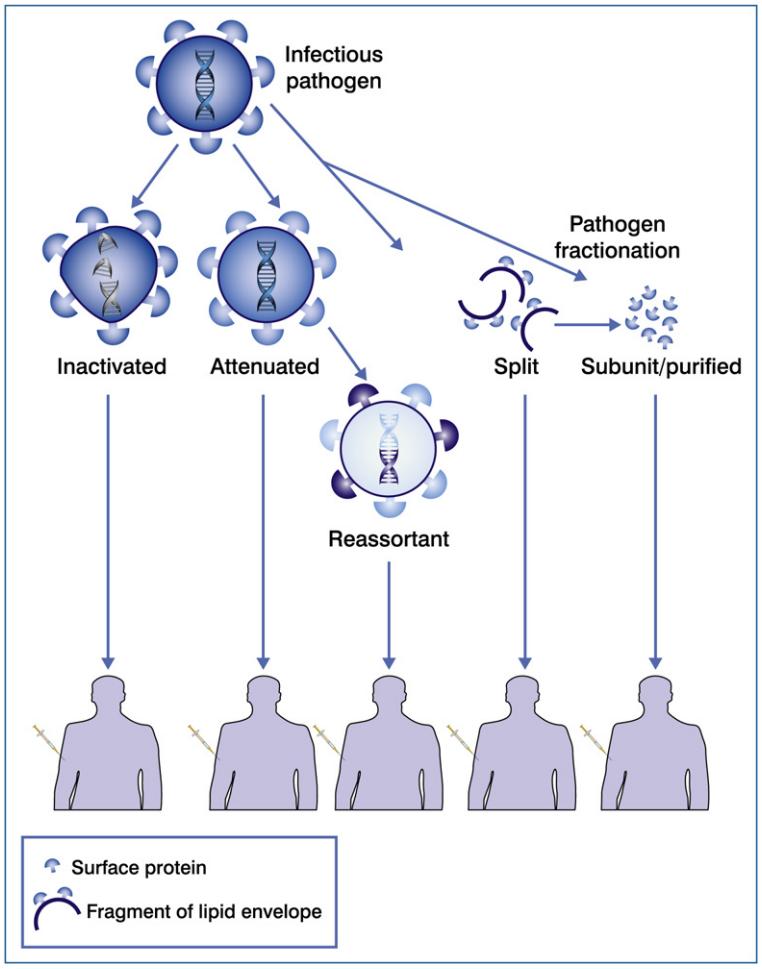


Figure 3.2 Approaches to vaccine antigen selection. Whole pathogen-based vaccines need to undergo attenuation or inactivation processes, while subunit vaccines rely on purified fractions of pathogens derived by physical disruption of whole organisms.

derived from at least two different strains of the same pathogen, and will express proteins derived from all component strains. Where whole-pathogen approaches are not feasible, other approaches, such as the use of split, subunit or recombinant antigens, will be considered. The choice of antigen is determined by what provides optimal outcomes in terms of safety and immunogenicity, and also by what is achievable by the standards of technology.

Further approaches to vaccine antigens may include recombinant DNA techniques (Figure 3.3), where the gene encoding the antigen is isolated and either expressed and purified from a protein-production system (eg yeast or insect cells) (Figure 3.3, panel A), or is expressed directly by the vaccine recipient following injection of an engineered *plasmid* (Figure 3.3, panel B) or a live vector (Figure 3.3, panel C). DNA-based candidate vaccines are in the earlier stages of development compared with their pathogen-based counterparts.

An experimental approach to vaccine peptide synthesis involves the direct joining of short fragments of peptides (molecules consisting of two or more amino acids) to form a complete antigen containing multiple *epitope*s from pathogen proteins (Figure 3.4). This can then be purified out from any truncated or incorrectly formed peptide segments, giving a pure and highly specific antigen. The benefit of such an approach is that it facilitates the generation of recombinant peptides that contain elements of antigenic proteins' conformational epitopes in a concatenated form (recognised by B cells) and linear epitopes (recognised by T cells).

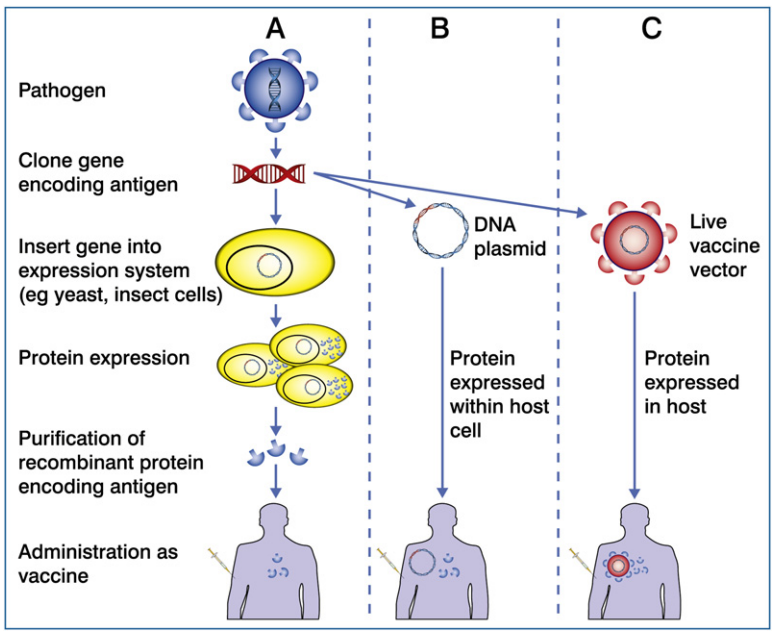
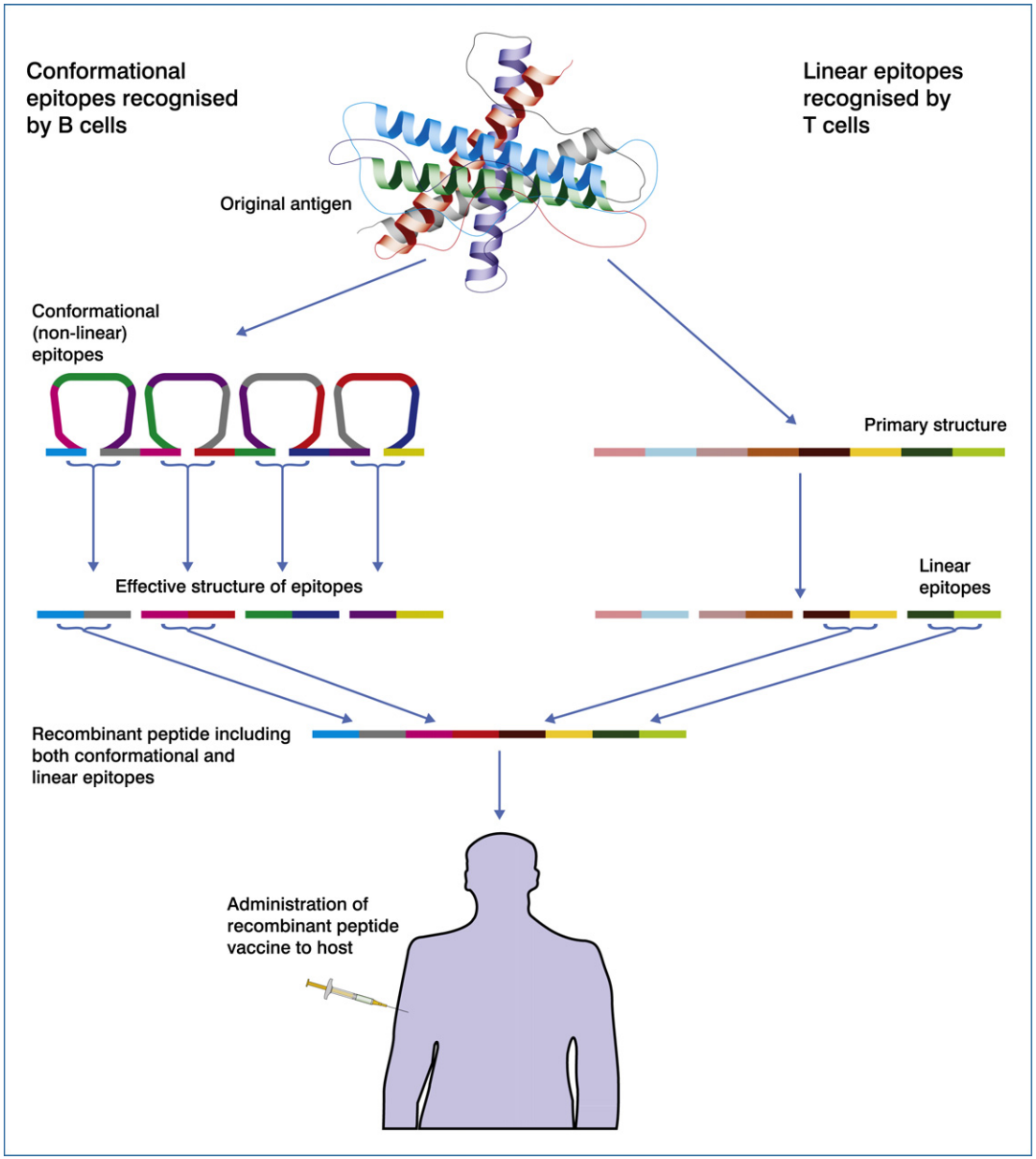


Figure 3.3 Recombinant/DNA approaches to vaccine antigens. Protein antigens are produced using recombinant DNA technology, where the DNA sequence coding for the antigenic protein is inserted into an expression system that is then able to produce large quantities of that specific antigen *in vitro* (panel A) or following administration to the host, eg using a DNA plasmid (panel B) or a live vaccine vector (panel C) as the expression system.

Figure 3.4 **Peptide approaches to vaccine antigens**. Recombinant peptide technology facilitates the generation of vaccines containing peptides that combine linear and elements of non-linear epitopes. Such vaccines are capable of stimulating an immune response from both B and T cells.



In every circumstance, the principle is to keep the antigenic structure or component of the pathogen intact and to eliminate most or all of the irrelevant and especially reactogenic features. DNA vaccines move the concept a step further, by using only selected genetic material from the pathogen, contained within an 'expression cassette' present within a small non-replicating piece of circular DNA. The antigen is then produced by cells of the vaccine recipient, which take up the injected DNA segment, allowing for direct production of the antigen *in situ* by the recipient.

Most of the possible approaches to the development of pathogen-derived vaccines are still in use, including whole inactivated and live attenuated, subunit and split pathogens, with and without *adjuvants*. DNA-based candidate vaccines are in earlier stages of development, although recent preclinical animal data for some pathogens have been promising.

Whole live, attenuated and killed/ inactivated pathogen vaccines

The most direct method for developing a vaccine is to use a whole pathogen, either killed/inactivated or attenuated (live but rendered harmless). These complete organisms are likely to contain all of the relevant pathogen-specific protein and carbohydrate antigens for effective *vaccination* and all or some of the innate defensive triggers that exist in the virulent pathogen. Moreover, live pathogen vaccines replicate and disseminate to their target tissue in a pattern similar to that occurring during a natural infection. The higher intensity of the innate immune responses, higher antigen content following replication and the more prolonged antigen persistence are the presumed mechanisms of how, generally, *live, attenuated vaccines* stimulate an effective and long-lasting immunity. Consequently, whole-pathogen vaccines can be highly effective and, if the pathogen can be grown quickly in cell culture, relatively easy to produce. A whole-pathogen vaccine can potentially be tested

and produced after identification and isolation of the pathogen without the development time associated with identifying and generating antigenic subunits, such as recombinant proteins or peptide epitopes. However, whole-pathogen vaccines are not a viable option for microorganisms which do not grow efficiently in cell culture, such as hepatitis B virus (HBV); or at all in *ex vivo* culture, for example *Mycobacterium leprae*. Several reasons why this approach may not be used either for specific pathogens or for vaccines intended for certain populations are discussed below.

LIMITATIONS OF THE WHOLE-PATHOGEN APPROACH

Loss of attenuation of live vaccines

Most live, attenuated vaccines undergo (limited) replication within host cells, which is problematic for some populations. If the restriction of growth of the attenuated organism is dependent, for example, on the presence of *$CD4^+$ T cells*, vaccination of people with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) may lead to serious infection/disease by the normally attenuated organism. For this reason, live, attenuated vaccines, such as the Bacille Calmette–Guérin (BCG) vaccine, are contraindicated in most immunocompromised people.

High reactogenicity

Some inactivated whole-pathogen vaccines are associated with high-frequency local or systemic *reactogenicity*. This reactogenicity is likely due to the potency of other microbial molecules that trigger the innate immune response. Two well-known examples of inactivated vaccines associated with high reactogenicity were the whole-cell pertussis vaccine and the first inactivated whole-virus influenza vaccine. In some countries, the reactogenicity profile of the vaccine produced a very low parental acceptance for infants and children, promoting the development of alternatives such as the subunit acellular pertussis vaccines and the split/subunit influenza vaccines.

Unwanted immune response

Unwanted and unexpected immune effects were observed with the first formalin inactivated respiratory syncytial virus (RSV) vaccine, developed in the 1960s. This inactivated whole-virus vaccine caused enhanced pulmonary pathology upon subsequent natural exposure of vaccinees to RSV compared with that seen in unvaccinated individuals (Kim et al., 1969). The root cause of this adverse effect is still not fully understood. One hypothesis is that the formalin treatment altered the structure of the protective antigens, resulting in the production of non-protective immunity. This hypothesis is supported by the finding that the vaccinees generated non-neutralising antibodies against the F and G proteins, which may have resulted in a delayed clearance of RSV from the lungs. More recently, a study showed that the exaggerated response might be due to low antibody *avidity* for protective epitopes (Delgado et al., 2009).

Risk of reversion

There is a small but calculable risk that attenuated pathogens, for example the oral polio vaccine, can reacquire the virulent genotype. In deletion mutants, this could occur through gene recombination with related microbes, replacing missing virulence genes in the vaccine strain. In 'culture-attenuated' mutants, which differ genetically from the natural pathogen because of the presence of sequence mutations that may involve a single nucleotide, random 'back mutations' could lead to the reactivation of silenced virulence genes. The history of use of live attenuated vaccines has shown that most of these vaccines are safe and that the risk of reversion is more theoretical than real.

Pathogen complexity

Pathogens are not only antigenically complex, but antigenic composition may change during their life cycle. Pathogens may also have complicated disease-causing pathways, involving multiple host tissues. For example, in some diseases, multiple different antigens are produced over different phases of the pathogen life cycle. These factors mean that different antigens would be protective against different stages of infection. A good

example of this is malaria, where the *Plasmodium* parasite undergoes several stages of development, each of which is antigenically distinct from other stages, and which occur in different anatomical locations. This makes it difficult to target all of the critical phases of the infective process using the whole pathogen from any single stage of development. This is one of the key challenges to producing an effective malaria vaccine. (It is not the only challenge as the immunodominant antigenic site is also subject to 'segment' mutation as different protein 'cassettes' are inserted at this site.)

Latency and immunoevasion

Some pathogens exist in a latent state within the host, often for the life of the host, or may be protected or hidden from the immune system and are, therefore, not available to the vaccine-induced immune response. *Latency* is a feature of bacteria, such as *Mycobacterium tuberculosis* (the causative agent of tuberculosis), and herpesviruses, such as cytomegalovirus (CMV), varicella zoster and herpes simplex viruses. In addition, some pathogens produce virulence factors that actively suppress or subvert host immunity, for example CMV produces proteins that can subvert or evade killing of infected cells by *natural killer cells*. In this case, vaccine formulation should consider alternative options to a whole-pathogen approach, to try to improve on nature.

Reduced immunogenicity

Research in antigen development has been driven by the reduced *immunogenicity* sometimes observed with highly attenuated or killed pathogen antigens. The procedure for attenuation or inactivation of the pathogen may remove vital defensive triggers, but could also remove/alter essential protective *immunogenic* components (epitopes) present in the intact pathogen, in which case the remaining antigens may not induce immune responses that protect the vaccine recipient against the live pathogen. An example of this is the live attenuated Towne vaccine strain of CMV which, although providing some protection against CMV disease in certain settings, is actually less protective than immunity that is

acquired naturally following recovery from CMV infection (*natural immunity*). This strain may have been over-attenuated by multiple (>125) passages through human cell culture, rendering it suboptimally efficacious as a vaccine.

Overall, however, when used in vaccines, whole live, attenuated pathogens are highly immunogenic, since both antigenic structures and defensive triggers, which activate the *innate immune system* (see *Chapter 2 — Vaccine immunology*) are present. Some of the relative advantages and disadvantages associated with live, attenuated and killed/inactivated vaccines are summarised in Table 3.1.

In order to address the above limitations of using a whole-cell pathogen approach, research has moved to define individual, or combinations of, pathogen-derived components that could be used instead as vaccine antigens. As mentioned previously, one of the first reasons to look for sub-cellular components was prompted by the high reactogenicity of some older whole-pathogen vaccines. This search has produced a new category of vaccines, the so-called split/subunit vaccines.

TABLE 3.1. CHARACTERISTICS OF LIVE AND KILLED VACCINES

Live attenuated	Killed/inactivated
Examples: OPV, MMR, VZV, some influenza, BCG	Examples: IPV, HAV, whole-cell pertussis
Mimic the natural infection and retain most defensive triggers/immunogenic elements; however, may retain immune evasion factors	Usually require adjuvants due to reduced immunogenicity/ missing defensive triggers
Strong priming usually achieved with 1—2 doses	Multiple doses usually needed for priming
Long-term persistence of immunity	*Booster* doses may be needed to maintain long-term immunity
May induce some mild disease symptoms	Do not induce disease symptoms
Rare revision to virulence; unsuitable for immunocompromised patients	No risk of reactivation, non-infectious
Potential for immunological interference with other live vaccines	Low risk of immunological interference
Less stable over time, heat labile	Relatively stable over time, better resistance to cold chain deviation
Response affected by recent administration of blood/blood-derived products or presence of maternal antibody in an infant	Generally not affected by administration of blood/ blood-derived products

OPV, oral polio vaccine; MMR, measles, mumps and rubella vaccine; VZV, varicella zoster virus vaccine; BCG, Bacille Calmette—Guérin (against severe forms of tuberculosis); IPV, inactivated polio vaccine; HAV, hepatitis A virus vaccine.

SPLIT-PATHOGEN/SUBUNIT ANTIGENS

Split-pathogen and *subunit antigens* are derived from physical separation and/or fractionation of the whole pathogen into smaller components with pieces of the viral envelope and surface antigens present in the antigen mix. There are various means of achieving this, including mechanical and chemical disruption. Among licensed vaccines, the majority use a subunit approach; influenza vaccines are currently the only vaccines to use a split-pathogen approach.

The toxoid-based vaccines of the early 20th century were the first subunit vaccines, although they were based on generating antibody to a disease-causing product of the pathogen rather than a structural component of the pathogen. Tetanus and diphtheria toxoid vaccines are designed not to prevent infection, but to elicit antibodies that bind and neutralise the bacterium's key *exotoxin*, since the toxins are responsible for the clinical symptoms of the disease.

More complete vaccine protection may be afforded using a combination of different subunit antigen components. Some acellular pertussis vaccines that comprise several subunit antigen components (eg pertussis toxoid, pertactin, filamentous haemagglutinin [FHA]), each of which provides limited protection, have demonstrated that multiple subunits can be combined to create an efficacious, well-tolerated vaccine.

DEFINING SUBUNIT ANTIGENS

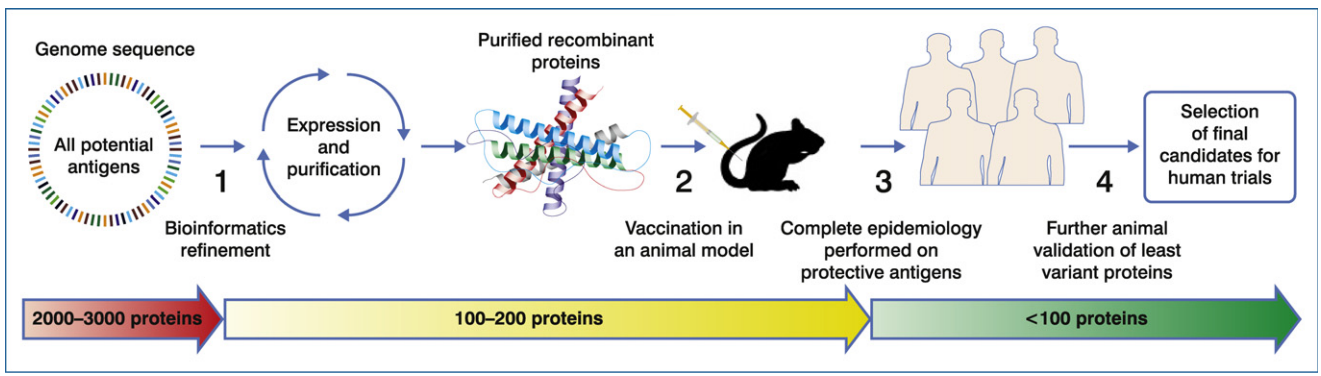
Purified subunits are antigenic proteins or polysaccharides, isolated from viral or bacterial structures and components. There are two broad approaches to determine which subunit antigens should be included in a vaccine. The classical approach is to study, in detail, the relationship between a pathogen and its host in order to identify the key virulence determinants that the pathogen requires for host entry, survival and/or dissemination to cause symptomatic disease. By mutating/deleting the genes encoding these virulence determinants and retesting the mutant pathogen in

an infection model, the importance of the individual determinant can be established.

The individual virulence determinant identified by the molecular postulates (which can be a protein or carbohydrate, eg capsule polysaccharide) is then purified and tested as a possible vaccine antigen.

An alternative approach is based on identifying the type of pathogenic structures that are most likely to be important immunogens according to their structural signature or physical location within the pathogen. Surface proteins of the viral envelope or viral capsid proteins can be good antigen candidates, although this is not so straightforward when the pathogen is a bacterium or a parasite, which may possess many hundreds of surface or secreted proteins. Advances in immunology, molecular biology and, especially, genomics and *bioinformatics* have made it possible to identify individual antigenic structures through computer-based searches of the pathogen's genome. This technique is known as '*reverse vaccinology*' (Figure 3.5).

Figure 3.5 Reverse vaccinology approach. In the search for vaccine candidates, the first level of refinement from all genes in the pathogen genome to genes encoding putative surface-exposed or exported proteins is algorithm based, using prediction software (1). Identified vaccine candidates are expressed in recombinant form, purified and used as a vaccine in a suitable animal model (2). Complete epidemiology is performed on the protective antigens in a search for antigenic variation (3). Invariant/least variant proteins or combinations thereof are further validated in animal models before moving into human clinical trials (4).



Starting with the genome of a pathogen, bioinformatics technology can identify genes that encode proteins with sequence characteristics, which suggest they are secreted or expressed on the surface of a pathogen. These genes can be isolated (ie the genes are 'cloned') and the proteins expressed in recombinant form in appropriate cells in culture. The proteins can then undergo testing as vaccine antigens in animals, singly or in pools. Those that are most immunogenic, or which stimulate protection in animal models, are selected for further laboratory development and preclinical testing. The technique may also require the testing of a large number of potential vaccine antigens that must be evaluated in a validated testing system, eg using an animal model that predicts how humans will respond. As additional checks, sera from the animal model or infected humans can be used to test *in vitro* neutralisation of virulence, ie to authenticate folding of the recombinant protein, or in passive transfer experiments to show that protection is antibody mediated, ie to define the *correlate of immunity/protection*.

Limitations of the subunit approach

A limitation of the subunit approach is that a cell culture-synthesised protein may not correctly form the three-dimensional structure that it assumes in the host, and may not induce protective antibodies. In addition, subunit vaccines often elicit weaker antibody responses than other types of vaccines, because of the lack of innate defensive triggers that drive the innate immune system.

Carbohydrate residues on antigenic proteins influence antibody binding, however, bacterial expression systems do not usually glycosylate recombinant proteins in a manner comparable to mammalian cells. Expression systems are, therefore, being continually improved to allow the production of glycoproteins that more accurately resemble a pathogen protein's native conformation.

The characteristics of split and subunit vaccine approaches compared with whole-pathogen approaches are provided in Table 3.2.

TABLE 3.2. CHARACTERISTICS OF SPLIT AND SUBUNIT VACCINE APPROACHES

Highly focused, specific response
Reduced immunogenicity and potential for escape mutants (pathogens that develop mutations resistant to the immunological protection conferred by vaccination)
Non-infectious
Low reactogenicity
No or limited availability of innate defensive triggers
Acceptable tolerability
Adjuvants normally required to compensate for lower immunogenicity
Synthetic production may be possible, facilitating supply (compared with whole pathogen)

HIGHLY SELECTED AND PURIFIED SHORT PEPTIDE ANTIGENS

Antigens generated by recombinant DNA technology

Improvement of industrial processes and sophisticated analytical methods allow us to take the concept of subunit vaccines a step further. HBV and human papillomavirus (HPV) vaccines are concrete examples of this approach. Specific antigenic proteins can be produced by recombinant DNA technology for viruses such as HBV and HPV that do not grow in cell lines. This approach also optimises the efficiency of the manufacturing process and the purity of the antigen (Figure 3.6). The gene encoding the specific protein of interest can be inserted into an expression system, eg a baculovirus, which is used to infect insect cells, or into yeast cells. Scalable production systems, such as yeast or baculovirus/insect cells, produce large quantities of the recombinant protein, which is harvested and purified. As the pure antigen is not accompanied by any of the elements that activate the defensive triggers of the innate immune system that would be present in the native pathogen, this approach results in an antigen that is well tolerated, but usually requires the addition of an adjuvant in order to achieve high immunogenicity and long-term protection.

A peptide antigen approach represents an additional step to the protein antigen approach. Peptide antigens may prove beneficial in the context of diseases where the pathogen evolves and protective antigens are numerous. In this setting, mixtures of different peptides known to be targets for protective immunity can be used more efficiently than producing many different full-protein antigens.

Innate 'defensive triggers' may be conserved molecular structures, such as repeating units of carbohydrate moieties, certain nucleic acid sequences, or molecules that are recognised by specialised pathogen receptors on innate immune cells and certain other cell types. The activation of immune defence mechanisms requires the presence of both antigen and defensive triggers to communicate the nature of the potential threat and to induce adequate immune responses. These elements may be missing in subunit and recombinant vaccine antigens and, for that reason, the addition of adjuvants and/or alternative ways of helping the antigens to stimulate the immune system are needed.

Direct synthesis of short peptide antigens

It is possible to identify and directly synthesise by various methods specific peptides that elicit adaptive immune responses. The peptides selected for vaccine development must contain epitopes that induce sufficient priming of naïve T cells to attain effective cellular and *humoral immunity*.

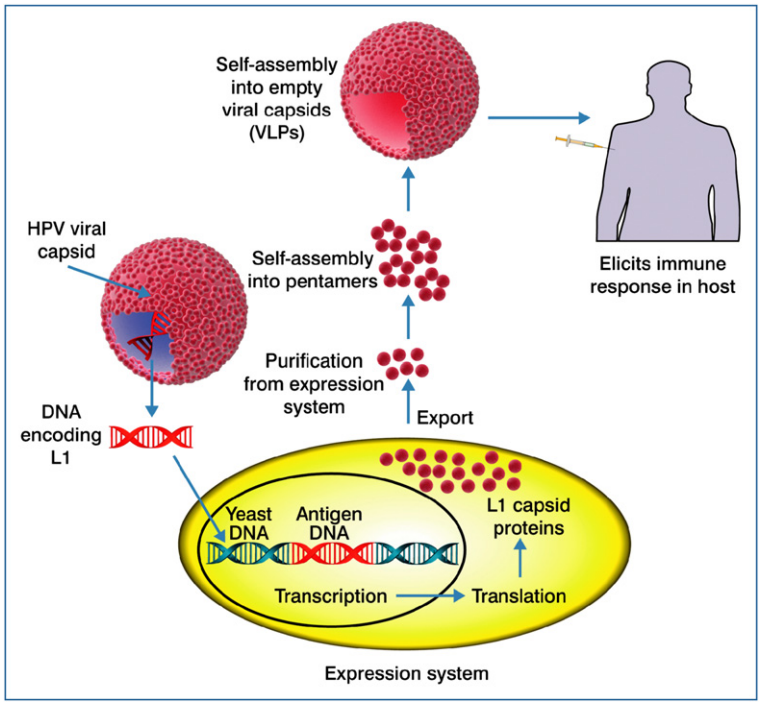


Figure 3.6 HPV L1 synthesis in a yeast expression system. Antigens can be produced using cellular expression systems. In the case of HPV L1 synthesis, the DNA encoding the L1 capsid protein from the HPV virus is inserted in the genome of yeast cells. Yeast cells then express high levels of recombinant capsid proteins. These are extracted from the yeast cell and purified. The L1 proteins aggregate to form pentamers, which then self-assemble into empty viral capsids. These VLPs form the antigenic component of HPV vaccines.
HPV, human papillomavirus; VLP, virus-like particle.

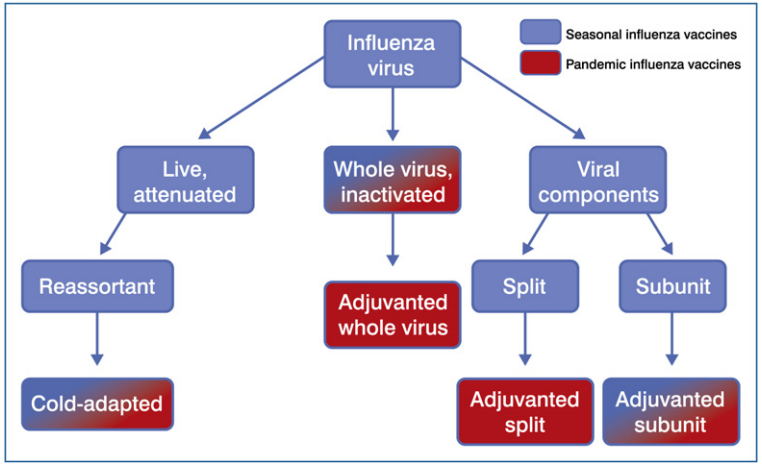


Figure 3.7 Types of influenza vaccine. The vaccines produced to combat influenza encompass most of the current approaches to antigen selection. Influenza vaccines therefore include attenuated and whole-virus antigens as well as split or subunit pathogen approaches.

CASE STUDY 1

Antigens for influenza vaccines: a variety of approaches to satisfy changing needs

Influenza vaccine technology encompasses most of the current approaches to antigen selection, including the use of whole viruses (Figure 3.7). The natural immune response to influenza viruses involves both humoral and cell-mediated immunity and the type-1 *interferon* response that is important for viral clearance. The humoral immune response is normally of more importance after viral clearance, and antibody responses associated with the *immunoglobulin* (Ig) G and IgA isotypes are important for protection against reinfection or infection with a new strain. Antibody against the haemagglutinin (HA) protein (a glycoprotein responsible for binding the virus to host cells) is considered the primary immune mediator of protection as this can inhibit virus binding to the epithelium, and thus block the early stages of infection. Antibody to the neuraminidase (NA) protein has also been considered as it can prevent cell-to-cell spread of the virus within the host. The evaluation of haemagglutinin inhibitory (HI) antibody *titres* has been used from the very beginning to assess influenza vaccine immune-protective abilities.

Seasonal influenza vaccines

The first seasonal influenza vaccines were based on live, attenuated viruses and produced in the 1940s. These vaccines were genetically prone to instability, resulting in variable degrees of attenuation and cases of influenza infection in some vaccinees. For this reason, this approach was abandoned in favour of inactivated whole formulations. Also first developed during World War II, killed whole-virus vaccines were immunogenic, but remained quite reactogenic, especially in children, where high rates of fever were recorded. This prompted the search for subvirion vaccines. Although whole-cell vaccines are still in use today in some countries, the majority of influenza vaccines manufactured over the last 30–40 years have been based on subunit and split-virus formulations, developed to minimise reactogenicity. These antigens consist of influenza fragments of varying degrees of purity. Some vaccines of this type are purified sub-virus particles (split vaccines), whereas others are based on highly selected and purified virus proteins or proteins produced from recombinant systems (subunit vaccines). The tolerability profile of these purified antigens is better than that with whole-pathogen vaccines, and their immunogenicity has been satisfactory. One dose of the vaccine is enough for the adult population, probably due to previous exposure to influenza, while two doses of split/subunit vaccines are needed in young children since most of them are naïve to influenza infections.

An ongoing challenge with seasonal influenza vaccines that continues to drive vaccine research is limited immunogenicity in the elderly. This is due to the natural process of

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immunological senescence – a declining ability of the immune system to mount effective immune responses with increasing age. One of the approaches to solving this problem is the use of adjuvants and two seasonal influenza vaccines, one adjuvanted with an oil-in-water emulsion and the other with a *virosome* (based on *liposome*), which became available in Europe in the 1990s. The adjuvanted vaccine improves immune responses in the elderly compared with the traditional non-adjuvanted vaccine.

Also in the 1990s, research on live, attenuated influenza vaccines experienced a resurgence as techniques, such as targeted gene deletions and *reassortment* of related strains, made it possible to produce vaccine strains with specific characteristics. These included cold-attenuated strains that were unable to replicate in the warm (core body temperature) environment of the lungs. This approach permitted the development of a trivalent cold adapted influenza vaccine first licensed in the USA in 2003 and currently approved for healthy children older than 2 years and adults less than 50 years of age. This vaccine, which is delivered intranasally, is updated with new reassortant strains each year to protect against seasonal influenza and is capable of inducing strong immune responses in children.

Pandemic influenza vaccines

Pandemic influenza presents unique challenges not seen with seasonal influenza, particularly the lack of pre-existing immunity in much of the population, which translates to the need for an even higher number of vaccine doses in a short space of time. In a pandemic setting, rapid protection following vaccination is desirable. Due to the special circumstances surrounding an influenza pandemic, vaccine manufacturers, regulatory bodies and health authorities approached the development of pandemic influenza vaccines in many ways. All known formulations were tested as pandemic candidate vaccines, including live attenuated and killed whole-virus vaccines, plus split-virion/subunit vaccines; the addition of adjuvants was also considered to reduce the amount of antigen in the vaccine, ie dose-sparing to maximise vaccine availability. Many of these vaccines were licensed for use in children, adults and the elderly during the 2009–2010 influenza pandemic. The adjuvanted split/subunit vaccines provided high levels of neutralising antibodies, exceeding the levels of antibody required by the licensing authorities in Europe and the USA with an important reduction of the antigen content and therefore offering the possibility to vaccinate more people. Overall, the live attenuated, whole killed and adjuvanted subunit pandemic influenza vaccines were immunogenic and well tolerated, and were made available in many parts of the world relatively quickly (see *Chapter 5 – Vaccine development*).

In response to the notion that influenza vaccines cause influenza-like symptoms, randomised, blinded studies have been performed in which trial volunteers were divided into two groups: influenza vaccine and placebo (Nichol et al., 1995). The only differences in symptoms between groups were increased soreness in the arm and redness at the injection site among those who received the influenza vaccine. There were no differences in terms of body aches, fever, cough, runny nose or sore throat.

Building and improving on nature

We can use our understanding of immunology and the interactions between host and pathogen in order to manipulate antigens to make them more immunogenic for vaccines. This is especially relevant for weakly immunogenic antigens, such as macromolecules consisting of repeating structural units (eg polysaccharides), and antigens that are most immunogenic when presented as part of larger molecules.

CASE STUDY 2

Conjugation of polysaccharide antigens to broaden the immune response

Potent antigens tend to have several common properties; they are generally foreign to the host, contain protein to drive T helper responses, are of high molecular weight (ie are macromolecules) and chemically complex. The degree to which each of these characteristics is present in a molecule determines how antigenic it is under given circumstances, and how effectively it induces immune responses. Some species of bacteria (such as all of those that cause meningitis) are enclosed within polysaccharides forming bacterial capsules. While they are large and complex macromolecules, polysaccharides are composed of repeating structural units that lack the ability to recruit T cells, which means that, chemically, they are simple molecules and hence are weakly antigenic. On exposure to encapsulated bacteria, the support for the B-cell response that should be provided by helper T cells, and which leads to immunological memory and highly potent response, is not optimally induced (Figure 3.8). This is because polysaccharide antigens do not contain T-cell epitopes and are not presented by *antigen-presenting cells* (APCs) to T cells. Bacterial capsular polysaccharides therefore primarily stimulate *thymus*-independent B-cell responses and are typically recognised by circulating mature B cells. These cells can produce short-lived responses, if the repeated polysaccharide antigen can cross-link the specific B-cell receptor Ig, but such responses are of low *affinity* and quickly wane.

Young children are particularly unresponsive to capsular polysaccharide antigens. The reasons for this are poorly understood, but may be due to the immaturity of the immune and

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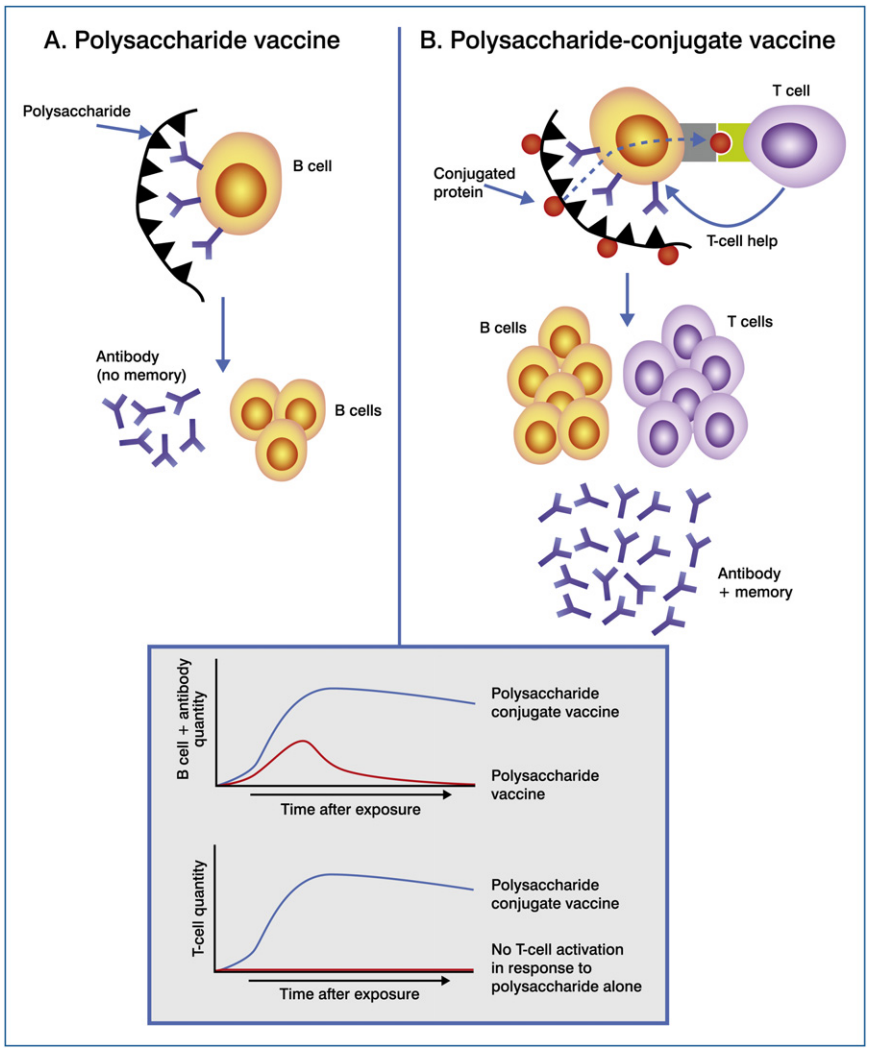
complement systems, and lack of a large enough pool of B cells to allow for clonal expansion (see *Chapter 2 – Vaccine immunology*). Although in adults there is an increased ability to respond to these antigens, the problem of frequent revaccination due to limited or absent induction of *immune memory* remains an important issue.

Bacterial infections by pathogens, such as *Haemophilus influenzae* type b (Hib), *Neisseria meningitidis* and *Streptococcus pneumoniae*, are responsible for the vast majority of bacterial meningitis cases. The polysaccharide capsules of encapsulated strains of these bacteria are a major virulence factor and define distinct serotypes within each species. Many of the most severely affected victims of these infections are young children, who cannot mount effective immune responses against encapsulated bacteria, and are at high risk of death or serious permanent consequences if not promptly treated with appropriate antibiotics. Vaccines against these pathogens based on purified polysaccharide components have a limited protective effect in adults and older children, but are poorly immunogenic in young children. Revaccination every few years is also needed regardless of age because of the vaccine's inability to induce immune memory.

The solution to this problem was the development of *conjugate vaccines*, where capsular polysaccharides are covalently linked to protein carriers known to be very immunogenic. This principle was first applied to Hib vaccine, and proved to be highly effective. Subsequently, other bacterial conjugate vaccines were developed for pneumococcal and meningococcal pathogens. Proteins used as conjugate carriers include tetanus and diphtheria toxoids, and protein D of non-typeable *Haemophilus influenzae*. The surface B-cell receptor of a polysaccharide-specific B cell binds to the polysaccharide component, triggering the first stages in the activation process. The protein toxoid components of these conjugates are then internalised and presented to specific T cells by B cells in the context of *major histocompatibility complex* (MHC) II molecules, resulting in B-cell help by the responding T cells. The activated B cells undergo antibody class switching to IgG and are then able to secrete high levels of anti-polysaccharide antibodies. The development of memory B cells specific for the polysaccharide antigen is also initiated – this is the key to providing long-term immune protection, as seen with the highly protective Hib, meningococcal and pneumococcal conjugate vaccines.

Figure 3.8 The principles underpinning polysaccharide conjugate vaccines. Polysaccharide antigens alone are weakly immunogenic, eliciting only a transient antibody response (panel A). Vaccines consisting of polysaccharides conjugated to proteins (panel B) elicit a greater antibody response and long-term memory including T cells, which are not generated with polysaccharide antigens alone. The immune response to such vaccines is illustrated in the charts within the boxed panel: the red lines illustrate the relatively low and transient production of B cells and antibodies in response to polysaccharide vaccines and the lack of a T-cell response, whereas polysaccharide conjugate vaccines produce a higher quantity of B and T cells and antibodies which are sustained for longer.



Virus-like particles and particulate antigens

Recombinant protein-DNA techniques make possible the production of highly pure proteins from pathogens. Several of these recombinant proteins, once harvested from the expression system and purified, aggregate in *particulate antigens*, which are more immunogenic than soluble antigens due to the way in which they interact with APCs. The enhanced ability of the innate immune system to recognise these types of structures is probably intrinsic rather than related to the specific antigen *per se*. This approach has been successfully applied in licensed vaccines for HBV and HPV, and in a candidate malaria vaccine currently in Phase III clinical trials.

An important consideration in vaccine design is defining what a vaccine should prevent – infection or consequences of infection, ie disease. The majority of vaccines prevent disease and not infection.

CASE STUDY 3

Recombinant proteins for the HBV vaccine antigen and beyond

The natural immune response to HBV involves the production of interferons by T cells and production of antibodies by B cells, in response to various components of the viral particle. Antibodies against the HBV surface protein are neutralising and protective against future infection, hence the levels of these antibodies are a serological correlate of protection. This protein (hepatitis B surface antigen [HBsAg]) was therefore selected as the antigen for the HBV vaccine. The antigen was initially derived from the plasma of chronic HBV carriers, but this plasma-derived vaccine presented certain issues from the perspective of supply depending on chronic HBV carrier donors, and also because of the risk (or fear of the risk) of transmission of blood-borne infections (although this was remote). It was not practical to use a classical subunit approach to developing non-infectious antigens, as HBV does not grow efficiently in cell culture. As a result, a recombinant protein approach was used to generate highly purified HBsAg for the vaccine (see Figures 3.3 and 3.6 for schematic representations of recombinant approaches to vaccine antigens).

The gene encoding HBsAg was sequenced to allow antigen production by recombinant DNA techniques in yeast expression systems. HBsAg was the first vaccine antigen to be manufactured through recombinant DNA technology, and represented a new and high degree of purity of a single protein antigen in a vaccine. This antigen was also the first to demonstrate that recombinant proteins can self-assemble into a particulate structure.

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The recombinant HBsAg protein adjuvanted with aluminium salt provides high levels of protection in immune-competent individuals and is a highly effective vaccine. However, in immune-compromised individuals, who respond less well to HBsAg vaccination, doubled dosages and multiple administrations are needed to ensure protective immunity, and some individuals fail to respond even after repeated immunisations. Alternative formulations were developed and studied, resulting in an HBV vaccine containing a new adjuvant combination: *Adjuvant System* (AS) 04 (see *Chapter 4 – Vaccine adjuvants*). The vaccine was developed specifically for use in pre-haemodialysis/haemodialysis patients, who respond poorly to the conventional vaccine and are at increased risk of HBV infection.

An additional application of the recombinant HBsAg has been the development of one of the more promising candidate malaria vaccines to date, RTS,S. This approach uses peptides from the malaria circumsporozoite (CS) protein (called RT), expressed as a hybrid matrix particle with the HBsAg and incorporated into a self-assembling complex – a presentation that enhances antigen recognition and processing by the immune system. This is delivered with a proprietary adjuvant combination, *AS01* (see *Chapter 4 – Vaccine adjuvants*). The RT portion includes both the CS repetitive B-cell (antibody-inducing) epitopes, as well as portions of non-repeat regions that had been identified as *T-cell determinants*. The candidate induces high levels of *cytokines* involved in Th1-biased T-cell activation. This candidate vaccine is now in Phase III trials after having shown protection in earlier clinical studies.

CASE STUDY 4

Recombinant antigens for vaccines against HPV and the VLP concept

Cervical cancer is a major killer of women worldwide caused by persistent cervical mucosal infection with oncogenic strains of HPV. HPV infections do not cause lysis of infected cells, thus avoiding initiation of inflammatory responses. The virus life cycle does not include a blood-borne phase, further limiting exposure of viral antigens to the immune system. Despite the attenuation of the immune response, however, the majority of naturally acquired HPV infections are cleared by cellular and humoral effectors, although natural immune responses following infection do not reliably protect against repeated HPV infection, particularly against different strains of HPV. Natural exposure (infection) therefore does not eliminate the risk of a subsequent HPV infection or the development of a persistent

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infection – a key step in the development of cervical cancer. Hence, in order to protect women throughout their lifetime, a vaccine must improve on natural immunity, eg immunity resulting from infection.

HPV presents a challenge for vaccination, which needs to induce a systemic adaptive immune response to a virus that enters and remains localised at the mucosal level. Preclinical studies showed that high levels of protection relied on the production of high levels of HPV-specific neutralising antibodies in the serum directed against the L1 surface protein, probably because they can transude/exude to the local cervical/vaginal mucosa. The successful HPV vaccine strategy that has been developed takes account of both the pathogenesis of infection and features of the host immune system. The antigen consists of a surface protein from HPV (L1 protein) that spontaneously assembles into empty capsid virus-like particles (VLPs). The protein is produced using recombinant DNA technology in yeast or insect cells (see Figure 3.6). The VLPs, which resemble the native virus, when combined with an adjuvant, are capable of inducing stronger and more protective immune responses than those resulting from infection.

Using this approach in the two licensed vaccines against HPV has provided an opportunity to protect against the major cause of cervical cancer.

The future of vaccine antigens

Targets of immune protection have been identified in many pathogens, knowledge of which is driving future vaccine design (Table 3.3). In addition to identifying targets of protection, many more challenges remain for vaccines, which are discussed in *Chapter 6 – Vaccines of the future*. These include tackling emerging pathogens and pathogens that display wide antigenic diversity, and populations with specific needs. In addition to identifying vaccine antigens against infectious diseases, in the last decade research has been intensified in order to find ways to develop vaccine-like immunotherapies against chronic disorders such as type I diabetes, Alzheimer's disease and cancer – where influencing the immune responses against specific antigens may play a role in prevention or cure. To address these challenges, new innovative methods of vaccine antigen design are being actively researched and developed.

TABLE 3.3. EXAMPLES OF KNOWN TARGETS OF IMMUNE PROTECTION

Pathogen	Targets of immune protection
Influenza	HA, NA
Pertussis	Pertussis toxoid Pertactin FHA Fimbriae
HPV	L1
Tetanus Diphtheria	Toxoids
HBV	HBsAg
Hib Pneumococcus Meningococcus Typhoid	Polysaccharides of the surface capsule of specific types

HA, haemagglutinin; NA, neuraminidase; FHA, filamentous haemagglutinin; HPV, human papillomavirus; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; Hib, *Haemophilus influenzae* type b. Every effort has been made to verify the information in this table. The information included is not meant to be exhaustive but to give an overview of the subject matter.

Conclusion

Advances in fundamental sciences such as immunology, as well as cell biology, genomic and proteomic technologies, may offer new avenues for vaccine development. The potential for increased pathogen attenuation, via elimination or the attenuation/modification/substitution of genes responsible for virulence, could allow us to selectively silence these key pathogenic determinants, while retaining the immunogenic and innate defensive signals. Broader application of reverse vaccinology may also lead to rational selection of antigenic components based on the hypotheses and theories that attempt to understand the workings of the immune system, while eliminating deleterious pathogenic products, resulting in extremely pure antigens of greater immunogenicity. Many future vaccines are

likely to be based on adjuvanted recombinant/highly purified antigens, due to the pathogenic and antigenic complexities of the remaining unconquered infectious agents (including HIV, hepatitis C virus, RSV, *Mycobacterium tuberculosis*; see *Chapter 6 — Vaccines of the future*). Where protective mechanisms are known or can be predicted, we are increasingly able to selectively induce these, using the most appropriate approach as outlined in this chapter. Understanding mechanisms of action of natural defence-triggering molecules has stimulated the design of new vaccine adjuvants that mimic natural responses to infection (see *Chapter 4 — Vaccine adjuvants*). The advances in vaccine technology have initiated a future of novel and innovative vaccine designs based on new knowledge of the antigenic properties of pathogens and the ways in which a protective immune response might be induced.

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