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92 Vaccines

Alan R. Shaw, Mark B. Feinberg

Vaccines represent one of the most effective and cost-effective medical and public health achievements of all time.[1] Worldwide, vaccination programs are currently estimated to save over 3 million lives each year. In addition to having such a major beneficial impact on vaccine-preventable disease morbidity and mortality, the direct and indirect impacts of vaccination programs translate into economic savings of many billions of dollars each year. In what is considered to be one of the most significant medical successes of all time, a collaborative and comprehensive vaccination campaign against smallpox resulted in the global eradication of the disease in 1979.[2] Similarly, efforts to eradicate poliomyelitis have made tremendous progress in reducing the global disease burden, and will hopefully soon overcome certain residual societal and programmatic obstacles to provide the second successful example of elimination of a major health threat by vaccination. Concerted global efforts to provide measles vaccine have resulted in the control and elimination of the disease in many countries, including substantial reductions in mortality in a number of developing countries where the residual disease burden is greatest. These and other examples provide clear evidence of the power of vaccines in favorably manipulating host immunity to confer dramatic public health benefits, at both the individual and population level.

As vaccines are administered to healthy individuals (often to entire age cohorts or populations), to prevent diseases caused by infectious agents to which they might be exposed in the future, they differ in important ways from pharmacologic agents that are used to treat individuals in whom a disease process is already manifest (or who display predispositions to disease). For this reason, vaccines are unique in the way that they impact on societies and in the way that societal commitment to vaccination determines their ultimate impact. As a result, vaccination efforts provide an informative window on challenges that need to be successfully navigated at the interface between scientific opportunity and societal capacity and commitment. Indeed, current limitations in realizing the full global potential of available vaccines relate more to existing inadequacies in health care financing and infrastructure (especially as they are manifest in developing countries), and the relative value that societies place on disease prevention, than they do to any inherent biological limitations of vaccines themselves. Fortunately, recent acceleration of new vaccine introductions in developing countries through public and private initiatives to build immunization infrastructure and provide funding of vaccine purchase offers hope that vaccines will one day be equitably available to all who need them.[3]

The importance of vaccines extends beyond their use as public health tools to include their role as drivers of immunologic discovery. The history of vaccine development is rich with immunologic insights that emerged from careful observations of how diseases spread in populations and how such spread differs in disease-naïve and experienced populations, as well as of how innovative experimental approaches revealed fundamental aspects of immune system function. The general concept of immunity induced by prior exposure to a disease (including its specificity and potential lifelong duration) was appreciated by the ancient Greeks. Use of the word 'immunity' itself dates to the 14th century when it was applied to describe the relative susceptibility and resistance of populations to plague. The subsequent successes of Edward Jenner and Louis Pasteur in the development of effective smallpox and fowl cholera immunization strategies, respectively, provided a foundation for modern immunology; Pasteur himself coined the term 'vaccine' in recognition of Jenner's use of vaccinia virus. Jenner's smallpox immunization studies also provided early experimental support for the concept of immune memory. Pasteur's efforts provided the first demonstration of the attenuation of pathogens by their propagation in culture (or by passage in nonnatural animal hosts), while Robert Koch demonstrated that killed pathogens could also engender immunity. The discovery of bacterial exotoxins by Emile Roux and Alexandre Yersin facilitated the discovery of antibodies and their potential use in passive immunotherapy with antitoxin antibodies by Emil von Behring and Shibasaburo Kitasato. These discoveries enabled the development of active immunization against diphtheria and tetanus using toxin–antitoxin mixtures. Paul Ehrlich's development of accurate methods for antibody quantitation made passive immunotherapy and active toxin–antitoxin immunization far more reliable and effective, and provided a stimulus for significant advances in immunologic theory. In each of these instances, vaccine development illuminated central mechanisms of immune system biology.

Vaccine development today has transitioned from an approach that was once largely empirical to one that is based on the hypothesis-driven application of techniques in molecular biology and immunology. Evidence for this synergy can be seen in recent studies of vaccine-elicited immune responses to illuminate primary and memory T- and B-cell responses in

humans, as well as the strong discovery stimulus provided by ongoing efforts to develop new vaccines for major infectious diseases for which vaccines are not currently available.

Vaccine development today faces a number of significant challenges. There exist tremendous public health needs to address major well-known pandemic diseases, including acquired immunodeficiency syndrome (AIDS), tuberculosis, and malaria, for which no vaccines currently exist and for which natural immunity does not provide a helpful guide for vaccine development. Furthermore, there exists a need to confront effectively newly emerging and re-emerging diseases, ranging from the well-known, but constantly changing, threats from influenza pandemics to the appearance of previously unknown zoonotic infections such as the coronavirus that causes severe acute respiratory syndrome (SARS). With changes in population density, mobility, and social constructs, along with alterations in the global climate, ecological circumstances, and the proximity of humans to animal reservoirs for previously confined infectious agents, the concept of new infectious agents entering human populations and spreading rapidly around the world is no longer novel. In confronting prevalent or newly emerging diseases, vaccines are looked to as the most promising line of defense. However, the speed at which new infectious disease threats have been shown to emerge and spread, and the fact that the pathogens that now need to be confronted may display tremendous genetic variability (e.g., human immunodeficiency virus (HIV)) or an identity that cannot be predicted in advance (e.g., avian influenza or agents like SARS) places unprecedented demands on the vaccine development process.

In addition to these new challenges, there remain unmet needs in the derivation of vaccines that can achieve the greatest public health benefit. These needs include the development of new ways to achieve more effective vaccine-elicited immune responses in neonates whose immune systems are immature (or are impacted by maternal antibodies) (Chapter 32) and in the elderly whose immune system function may be waning as a result of immune senescence (Chapter 33). Fortunately, the scientific foundation provided by basic and applied immunology and the use of new methods for pathogen identification, antigen discovery, vaccine production, adjuvant development, and novel vector derivation afford important opportunities for vaccine development and additionally present the possibility of improving on natural immunity.

Success in vaccine development will be predicated on continuing the historical synergy between advances in vaccine technology and basic immunologic discovery. Toward that end, this chapter focuses on preventive vaccines for infectious diseases and how they are developed. Although current routine vaccine recommendations are reviewed, given the active state of new vaccine introduction and evolving vaccine recommendations, as well as differences in recommendations in different countries, readers are encouraged to refer to up-to-date national resources for the most current information. While vaccine approaches are being actively explored to modify beneficially malignant and immunologic diseases (autoimmunity and allergy), these are beyond the scope of the current discussion.

IMPACT OF VACCINATION PROGRAMS

Unlike other medical interventions, vaccines confer benefits to both individuals and populations.[4,5] While individuals may be protected from infection or disease by vaccine-induced immune responses, decreasing the number of susceptible hosts in a population also helps break the chain of transmission that pathogens require to spread and persist in human populations by induction of 'herd immunity.' The benefits of herd immunity depend on achieving sufficiently high immunization rates in a population to impact pathogen transmission dynamics (including the potential for extinction of ongoing interhost transmission). The requisite level of vaccination coverage of a population needed to compromise pathogen spread significantly varies between pathogens, and is influenced both by vaccine efficacy (and its duration) and by the reproductive characteristics and infectiousness of the pathogen.

Analysis of the impact of vaccination programs in the USA provides an example of the beneficial impact of vaccines when used routinely and when high coverage levels are achieved.[6] As shown in Tables 92.1 and 92.2, vaccination programs in the USA dramatically decreased the annual morbidity of many vaccine-preventable diseases. In many instances, the disease burden from several vaccine-preventable diseases of childhood has been reduced by over 99% since vaccine introduction (e.g., diphtheria, tetanus, measles, mumps, rubella, and polio). The somewhat lower rate of decline of pertussis (the annual morbidity of which has been reduced by a nonetheless impressive 83%) relates to the limited duration of vaccine-induced immunity, which is estimated to wane within 5–10 years after childhood vaccination. It is anticipated that recent availability of pertussis booster vaccines for use in adolescents and adults will lead to significant further declines in pertussis morbidity. Even for diseases targeted by vaccines that have been in widespread use for less time (< 10 years), impressive decreases in disease morbidity have been seen (e.g., varicella, hepatitis A, and pneumococcal disease). In a notable recent demonstration of the population benefits of vaccines, introduction of the 7-valent pneumococcal conjugate vaccine resulted in a decrease of 73% in disease morbidity in children under 5 years of age within the first 5 years of its introduction. Interestingly, the rate of meningitis and bloodstream infections caused by antibiotic-resistant *Streptococcus pneumoniae* also fell by 81% in this age group. In a striking related finding illustrating how vaccines can impact pathogen transmission dynamics, rates of antibiotic-resistant pneumococcal infections also declined by 49% in individuals over the age of 65 who had not received the vaccine. Thus, direct protection by vaccination of children who represent a reservoir of infection provided, via herd immunity, significant indirect benefits to those who did not themselves receive the vaccine.

In addition to their benefits in preventing disease morbidity and mortality, routine vaccination programs are also impressively cost-effective. Evaluation in the USA of the impact of ten vaccines routinely given as part of the childhood immunization schedule (diphtheria, tetanus, pertussis, *Haemophilus influenzae* b (Hib), polio; measles, mumps, rubella, hepatitis B and varicella) found that more than 14 million cases of disease and more than 33 500 deaths were averted over the lifetime of the immunized birth cohort of children.[7] When the cost of the vaccination program was compared to the economic impact of diseases prevented, these vaccines alone are estimated to save nearly $10 billion each year. When including indirect economic benefits (such as the time parents take off from work to care for sick children), the annual savings to society exceed $40 billion. When 30 preventive services were ranked based on clinically preventable disease burden and cost-effectiveness, childhood immunization received the highest score.[8]

Progress in the development of new vaccines accelerated significantly towards the end of the 20th century, with the development of vaccines against diseases that were not previously preventable by vaccination, but also with the development of improved versions of existing vaccines.

Table 92.1 Comparison of 20th-century peak annual morbidity versus current annual morbidity: United States vaccine-preventable diseases

Disease	20th-century annual morbidity[1]	2005[2]	Decrease (%)
Smallpox	48 164	0	100
Diphtheria	175 885	0	100
Measles	503 282	66	> 99
Mumps	152 209	314	> 99
Pertussis	147 271	25 616	83
Polio (paralytic)	16 316	1[3]	> 99
Rubella	47 745	11	> 99
Congenital rubella syndrome	823	1	> 99
Tetanus	1314	27	98
Haemophilus influenzae	20 000	226[4]	99

[1]Source: Centers for Disease Control. MMWR 1999; 48: 242–264.
[2]Source: Centers for Disease Control. MMWR 2006; 55: 880–893.
[3]Imported vaccine-associated paralytic polio (VAPP).
[4]Type b and unknown (< 5 years of age).
Numbers indicate at- or near- record lows in 2005 (except pertussis).

Table 92.2 Comparison of pre-vaccine-era estimated annual morbidity versus current estimated morbidity: vaccine-preventable diseases, United States

Disease	Pre-vaccine-era estimated annual morbidity[1]	2005 Estimated morbidity[1]	Decrease (%)
Hepatitis A	117 333	19 183	84
Hepatitis B (acute)	66 232	15 352	77
Pneumococcus (invasive)			
All ages	63 067	40 325	36
< 5 years of age	16 069	4 400	73
Varicella	4 085 120	817 024	80

[1]Unpublished Centers for Disease Control data, reported November 2006.

Thus, the number of diseases that can be prevented by vaccines included in the US Centers for Disease Control and Prevention's (CDC) routine childhood and adolescent immunization schedules grew from seven in 1985 to 16 in 2007 (Table 92.3 and Fig. 92.1). Moreover, in the past several years, new vaccines have been introduced for adolescents and young adults (e.g., pertussis booster (Tdap), meningococcal conjugate, and human papillomavirus (HPV) vaccines), and older adults (e.g., Tdap and zoster vaccines) have shown that the value of vaccines extends across the human lifespan (Figs 92.1 and 92.2). New combination vaccines have been developed to increase the simplicity and acceptability of vaccination regimens, as well as to improve overall compliance with the recommended series of vaccines. Such combinations include either those that contain multiple inactivated or recombinant antigens (such as a combination diphtheria, pertussis, tetanus, Hib, and hepatitis B vaccine) or multiple live attenuated viruses (such as a combination measles, mumps, rubella, and varicella vaccine (MMRV)). The development of a combination vaccine is often more complicated than simply combining individual antigens, for when antigens are administered in combination, immunologic interference is sometimes seen. This necessitates titration of antigen combinations (and in the case of combinations of inactivated and/or

Table 92.3 Number of diseases prevented by vaccines included in the US Centers of Disease Control and Prevention's routine childhood and adolescent immunization schedules

Year	1985	1995	2007
Disease	Measles Rubella Mumps Diphtheria Tetanus Pertussis Polio	Measles Rubella Mumps Diphtheria Tetanus Pertussis Polio *Haemophilus influenzae* b (infant) Hepatitis B Varicella	Measles Rubella Mumps Diphtheria Tetanus Pertussis Polio *Haemophilus influenzae* b Hepatitis B Varicella Pneumococcal disease Influenza Meningococcal disease Hepatitis A Rotavirus Human papillomavirus
Number of vaccine-preventable diseases	7	10	16

recombinant antigens, adjuvant selection) to achieve immune responses that are not inferior to each of the antigens administered individually.

Despite their readily demonstrable public health impact, the value of vaccines is often not appreciated, for when vaccine programs are successful the diseases that they cause become less prevalent and may disappear. However, to prevent resurgence of an infectious disease that has been brought under control, vaccination programs need to be continued. The difficulties facing current efforts to eradicate poliomyelitis have demonstrated that failure to maintain high immunization coverage rates can lead to prompt re-emergence and spread of the disease. Even in developed countries, maintenance of strong immunization programs with high degree of coverage is needed where infectious diseases can travel with remarkable speed – and do so even before the extent of spread is evident.

PRINCIPLES OF IMMUNIZATION

The terms *vaccination* and *immunization* are often used interchangeably. However, vaccination specifically refers to efforts to induce protective immune responses by administration of a vaccine, whereas immunization more generically refers to interventions – either active or passive – that seek to confer immune protection. Active immunization describes the induction of immune responses by administration of a specific antigen or antigens, while passive immunization involves the administration of exogenous immunologically active substances (historically, antibodies present in sera obtained from immune individuals or animals) to confer temporary protection from an infectious pathogen or toxin. Although the approaches for passive immunization waned in the later half of the 20th century, the advent and increasing robustness of monoclonal antibody technology have led to a resurgence of interest in passive immunization.

THERAPEUTIC PRINCIPLES

Special attributes of vaccines

- Vaccines benefit both individuals and populations
- Vaccines represent one of the most effective and cost-effective public health innovations of all time
- To be most effective, vaccines need to be administered to the targeted cohorts of individuals in advance of when they might be exposed to the pathogen of interest
- Vaccination of a sufficient number of individuals in a population can, by induction of herd immunity, impact the transmission dynamics of pathogen spread in a population such that even unimmunized individuals are less likely to become infected
- With sufficiently high and prolonged immunization coverage, and depending on whether or not nonhuman reservoirs for pathogen persistence exist, it is possible to eradicate infectious diseases from human populations (as was accomplished with smallpox and is now being pursued for poliovirus)
- For many contemporary global health threats (e.g., human immunodeficiency virus (HIV), tuberculosis, malaria, and pandemic influenza), the development of effective vaccines is considered to represent the most promising strategy for public health protection
- Unlike drugs that are administered to individuals with (or at risk for) specific diseases, vaccines are commonly administered to healthy individuals. As a result, the risk-to-benefit ratio for vaccines requires that vaccines meet high standards of safety and tolerability

DEPARTMENT OF HEALTH AND HUMAN SERVICES • CENTERS FOR DISEASE CONTROL AND PREVENTION

Recommended Immunization Schedule for Persons Aged 0–6 Years—UNITED STATES • 2007

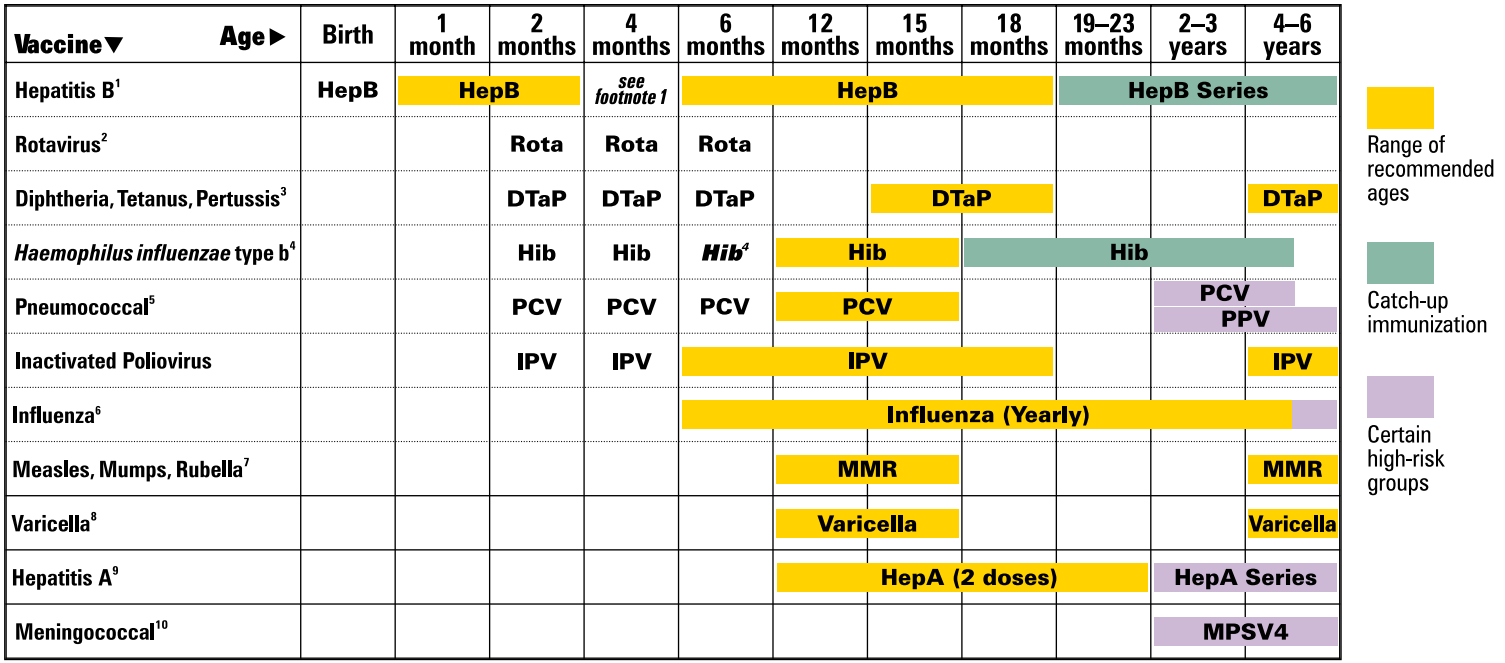

Fig. 92.1 Recommended immunization schedule for children and adolescents aged 0–8 years – USA, 2007. These schedules indicate the recommended ages for routine administration of currently licensed childhood vaccines (for children aged 0–6 years and for children aged 7–18 years), as of December 1, 2006 (published in MMWR 2007; January 5). Additional information is available at www.cdc.gov/nip/recs/child.htm. Any dose not given at the recommended age should be given at any subsequent visit, when indicated and feasible. Providers should consult the respective Advisory Committee on Immunization Practices (ACIP) statement for detailed recommendations. The Recommended Schedule for Persons Aged 0–18 is updated annually by the Centers for Disease Control and Prevention (CDC). Readers can find the most current vaccination recommendations from CDC at www.cdc.gov/nip/publications/acip-list.htm for official ACIP recommendations and www.cdc.gov/nip/recs/provisional_recs/default.htm for provisional ACIP recommendations.

Continued

DEPARTMENT OF HEALTH AND HUMAN SERVICES • CENTERS FOR DISEASE CONTROL AND PREVENTION

Recommended Immunization Schedule for Persons Aged 7–18 Years—UNITED STATES • 2007

Vaccine ▼ / Age ▶	7–10 years	11–12 YEARS	13–14 years	15 years	16–18 years
Tetanus, Diphtheria, Pertussis[1]	see footnote 1	Tdap	Tdap		
Human Papillomavirus[2]	see footnote 2	HPV (3 doses)	HPV Series		
Meningococcal[3]	MPSV4	MCV4	MCV4	MCV4[3]	
Pneumococcal[4]	PPV				
Influenza[5]	Influenza (Yearly)				
Hepatitis A[6]	HepA Series				
Hepatitis B[7]	HepB Series				
Inactivated Poliovirus[8]	IPV Series				
Measles, Mumps, Rubella[9]	MMR Series				
Varicella[10]	Varicella Series				

Range of recommended ages

Catch-up immunization

Certain high-risk groups

Fig. 92.1 continued.

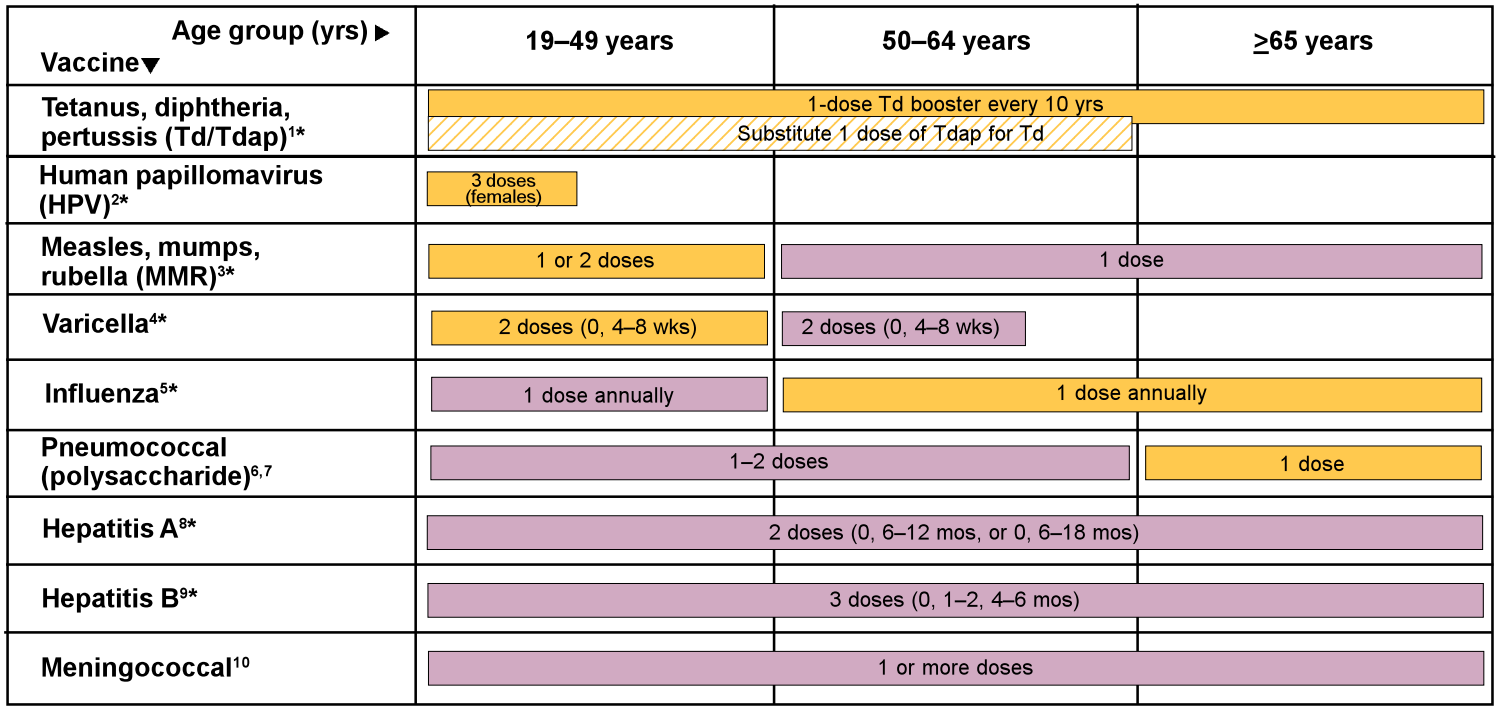

Fig. 92.2 Recommended immunization schedule for adults – USA, 2007. This schedule indicates the recommended age groups (A) and medical indications (B) for routine administration of currently licensed vaccines for persons aged 19 years or older, as of October 1, 2006 (published in MMWR 2006; October 13). For detailed recommendations on all vaccines, including those primarily for travelers or that are issued or updated over time, consult the manufacturers' package inserts and the complete statements of the Advisory Committee on Immunization Practices (ACIP) (http://www.cdc.gov/nip/publications/acip-list.htm). Recently issued, provisional ACIP recommendations can be found at www.cdc.gov/nip/recs/provisional_recs/default.htm.See url for footnotes to the figure.

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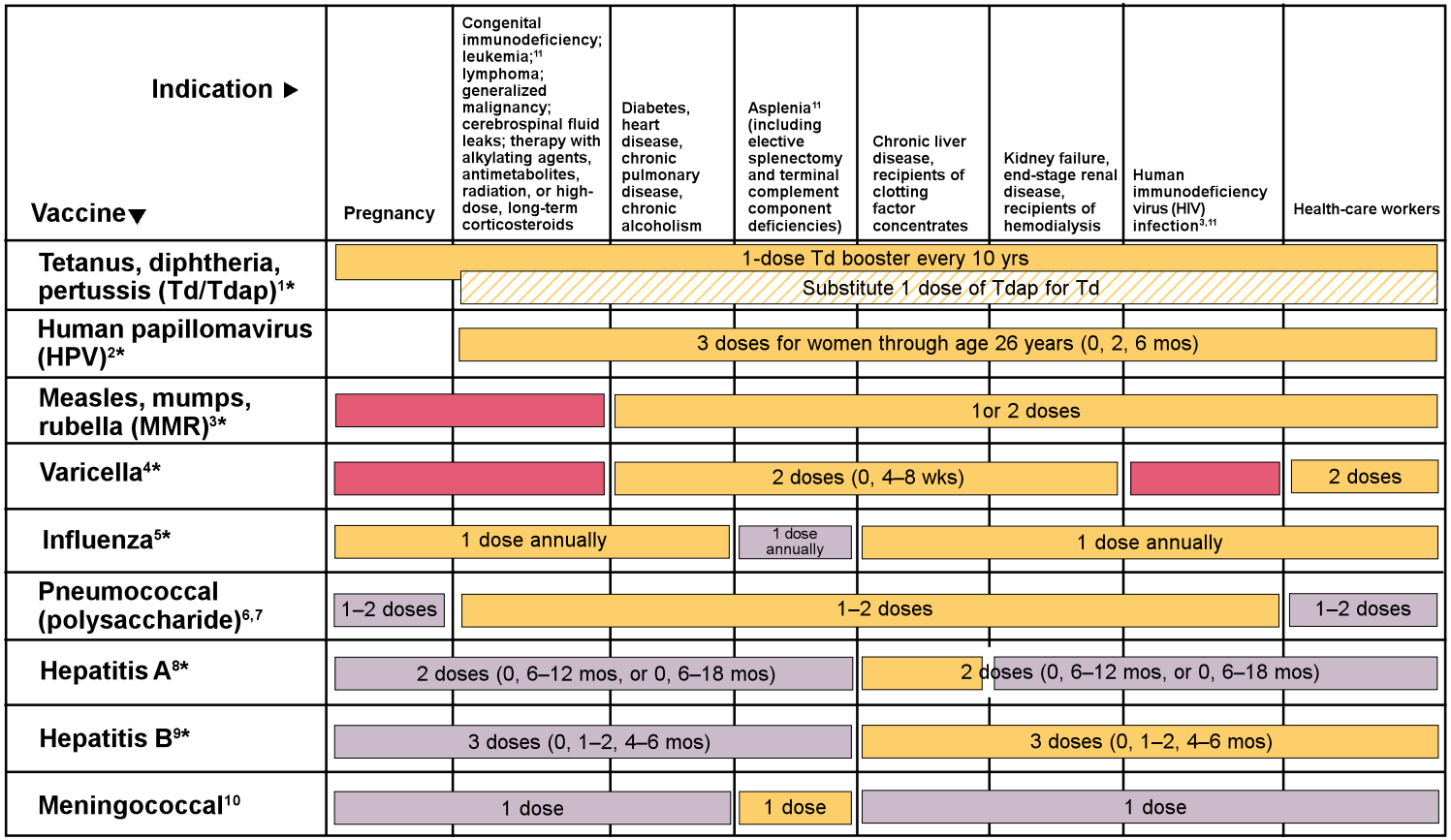

Fig. 92.2 continued.

Vaccines seek to engender immune responses similar to those that confer immunity to re-infection in individuals who experience (and survive) natural infection with a given pathogen. In lieu of formal demonstration of a specific type of antibody or cellular immune response that contributes to prevention or accelerated clearance of an infection, most often vaccine efficacy is demonstrated first in the course of a placebo-controlled trial. In some instances, specific immune effector mechanisms, such as a specific level or type of antibody response, can be identified that correlate with immune protection. In this case, the 'correlate of immunity' provides a benchmark against which similar vaccines can be compared.

In the case of most inactivated vaccines, subunit vaccines, and recombinant vaccines that produce antibody responses, but generally meager CD8 T-cell responses, it is likely that humoral immune responses are the primary or sole protective immune mechanism. In the case of live attenuated vaccines that induce both cellular and humoral immune responses against the pathogen, it is likely that both arms of the immune system act in concert to confer immunity. However, the actual mechanisms of immune protection induced by either a natural infection or a vaccine are generally not understood in detail for many infectious diseases.

Similarly, although vaccines depend on the induction of immunologic memory, the magnitude, character, and duration of immune memory differ between vaccines, as can the actual mechanism of immune protection. For certain vaccines, such as those that protect against bacterial diseases induced via production of toxins (e.g., diphtheria or tetanus), protection induced by toxoid-based vaccines is clearly dependent on persistent antibody (IgG) and memory B-cell responses, ensuring that sufficient antitoxin antibodies are present at the time of toxin exposure to inactivate and clear the toxin. In other cases, such as long-lived protection against hepatitis B, if sufficient levels of antibodies are achieved in the initial immunization period, even hosts who may with time lose detectable levels of antibody responses remain protected.[9] In this instance, given the relatively long incubation period of hepatitis B, memory antiviral B-cell responses induced by the vaccine can be activated, facilitating neutralization and clearance of the infection before clinical disease is manifest. Although it is popularly believed that vaccines confer protection by inducing 'sterilizing immunity' – wherein an infectious agent is blocked from even infecting one cell in an exposed host – this is clearly not the case for a number of vaccines. For example, the inactivated poliovirus and live attenuated rotavirus vaccines do not prevent some degree of local replication of their pathogenic counterparts in the gastrointestinal tract of exposed hosts. However, they are both effective in preventing clinical disease. In the case of poliovirus vaccine, this is mediated by elicitation of antibody responses that block dissemination of the infection to the central nervous system; while in the case of rotavirus, as yet unidentified immune effectors limit local virus replication so that significant gastrointestinal damage does not occur following infection.[10, 11]

The major types of vaccines licensed for use include live attenuated organisms, killed or inactivated organisms, subunit vaccines consisting of purified (or partially purified) components of an organism, and subunit vaccines produced by recombinant DNA technologies.

LIVE ATTENUATED VACCINES

The use of live attenuated vaccines dates back to the early work of Jenner and Pasteur on smallpox and fowl cholera vaccines, respectively.[12, 13] The fundamental concept of live attenuated vaccines is to mimic the effective host immune responses that follow natural infections. Most live attenuated vaccines currently in use were derived by propagation of initially pathogenic organisms in culture on cells from different (nonhuman) species, or at nonphysiologic temperatures, for prolonged periods. Driving pathogen evolution in culture to select for variants adapted to growth in heterologous cell types *ex vivo* often leads to the derivation of pathogen variants that grow poorly *in vivo* in humans and are unable to cause clinical symptoms.

Vaccines developed via this approach include those used to prevent a number of viral and bacterial infections, including yellow fever, measles, mumps, rubella, polio (the 'Sabin vaccine'), varicella-zoster (used both for the prevention of chickenpox and shingles) and rotavirus (one version of the available vaccines), tuberculosis, and cholera. More recent technologies being applied to live attenuated vaccine development include the application of reverse genetic strategies (Fig. 92.3) and those involving genetic reassortment with attenuated viral variants, as have been used to develop polyvalent live attenuated vaccines against influenza and rotavirus (Fig. 92.4).[10, 11]

The live attenuated vaccines currently in use are highly efficacious (> 90%) and protection is frequently durable. The efficacy of many live attenuated vaccines likely reflects the ability of the attenuated vaccine to replicate within vaccinated hosts, and to expose the immune system to pathogen-derived antigens in a manner that closely resembles the nature, location, and effects of natural infection. Because live attenuated vaccines replicate within immunized individuals, they can induce both cellular (CD4 and CD8) and humoral (B-cell) effector responses and immunologic memory. In addition, as the live attenuated vaccines likely activate the host innate system in a manner similar to their pathogenic parents, they provide inherent adjuvant effects in augmenting adaptive immune responses.

A key consideration in the development of any live attenuated vaccine relates to the relative balance between the ability to induce sufficient immune responses *in vivo* to confer protection (often associated with level of preserved replicative ability *in vivo*), and the ability to cause symptoms (which may also relate to the extent of *in vivo* replication). As such, an effective but also safe and well-tolerated vaccine needs to strike a specific balance between level of attenuation and level of immunogenicity. In addition, depending on the nature and number of genetic mutations responsible for the attenuated phenotype, a potential risk of reversion to a pathogenic form exists for certain vaccines. For most live attenuated vaccines, this has not been observed to be a problem in clinical practice – likely because the attenuating mutations are sufficiently numerous or genetically stable. One vaccine where reversion to pathogenic form was seen involved specific components of the live attenuated oral poliovirus vaccine (OPV; the 'Sabin vaccine'). In this instance, vaccine reversion to wild-type was shown to lead rarely to cases of paralytic polio (approximately one case per million doses administered).[14] Based on these observations and the elimination of endogenous polio transmission in many developed countries, the inactivated polio vaccine (IPV; the 'Salk vaccine') was substituted for OPV. However, in light of a favorable cost–benefit ratio, high degree of efficacy, and ease of administration, OPV continues to be the mainstay of polio vaccination efforts in developing countries.

KILLED OR INACTIVATED ORGANISMS

The use of physical or chemical methods to kill or otherwise inactivate a pathogenic organism represents a second major approach to vaccine production.[15, 16] In most cases, treatment with chemical agents such as β-propiolactone and formaldehyde is used to eliminate pathogen

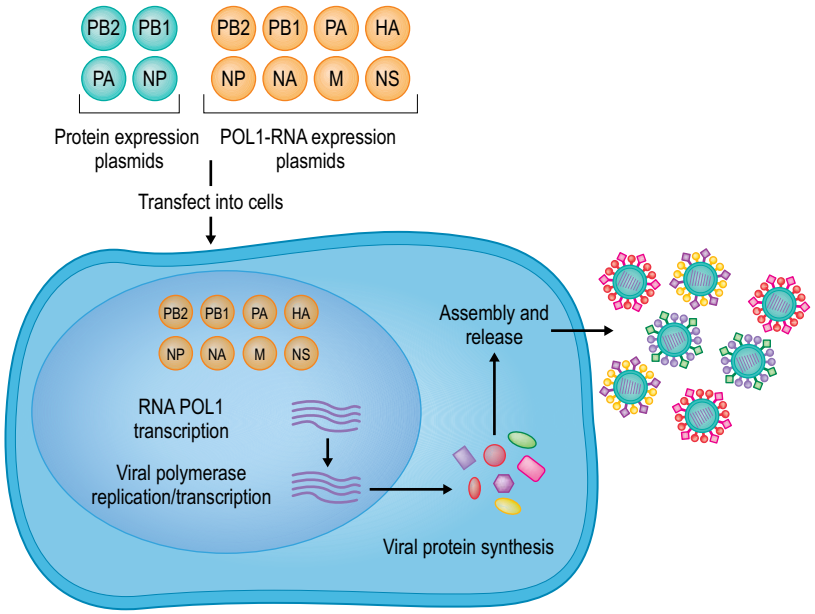

Fig. 92.3 New vaccine strategies: reverse genetic approaches. The term 'reverse genetics' refers to the use of recombinant DNA methods to generate infectious viruses possessing genomes derived from cloned cDNAs. Such cDNAs can be modified to study the impact of specific genetic modifications to viral phenotype, providing a new approach for the generation of live attenuated vaccines via either introduction of targeted mutations or, in the case of segmented viruses, the preparation of vaccines via genetic reassortment (see Fig. 92.4). Reverse genetic methods provide promising tools for the study and defined manipulation of both nonsegmented and segmented negative-strand RNA viruses (such as the respiratory syncytial virus (RSV) and influenza viruses, respectively). The use of reverse genetic strategies to generate infectious viral progeny from cloned cDNAs is shown above. Influenza virus genomes are comprised of eight single-stranded (negative-sense) RNA segments. Initiation of influenza virus RNA (vRNA) transcription from negative-sense genomic RNAs, and the replication of the virus genome, depends on the viral ribonucleoprotein (RNP) complex (which includes viral RNA, the nucleoprotein (NP) and three polymerase proteins (PB1, PB2, and PA)). To generate infectious influenza virus from cDNAs of the vRNA genome segments, cells are cotransfected with all eight segments of vRNA under the control of RNA polymerase promoters. Cellular polymerase I (pol 1) synthesizes vRNAs that are then replicated and transcribed by the viral polymerase and NP proteins that comprise the RNP complex. Reverse genetics strategies are expected to facilitate the generation of novel flu vaccines by enabling preparation of well-defined vaccine preparations comprised of donor 'backbone' viral segments (see Fig. 92.4) that harbor specific attenuating mutations with vRNA segments encoding the hemagglutinin (HA) and neuraminidase (NA) proteins obtained via reverse transcription of vRNA genes from circulating viruses (including those prepared from pandemic strains that may be difficult and/or unsafe to propagate in large manufacturing scale). (Adapted from Marsh GA, Tannock GA. The role of reverse genetics in the development of vaccines against respiratory diseases. Exp Opin Biol Ther 2005; 5: 369–380, with permission from Expert Opinion.)

infectivity. While this approach has the benefit of presenting most of a pathogen's antigenic repertoire to the immune system of the immunized host, it can only be used in instances where the inactivated pathogen does not possess constituents that would confer significant toxicity. Vaccines based on killed pathogens are believed to exert their protective effects via elicitation of pathogen-neutralizing antibodies and the induction of memory B-cell responses (likely in concert with CD4 T-cell memory). However, because inactivated pathogens cannot accomplish *de novo* synthesis of pathogen-derived gene products in antigen-presenting cells (APCs), they do not typically induce CD8 T-cell responses (Chapter 6). In addition, killed vaccines are generally less immunogenic than live attenuated vaccines. As a result, they are commonly administered with an adjuvant (most often alum: see section on Adjuvants, below) to augment their immunogenicity. A number of viral and bacterial vaccines currently in use are killed/inactivated vaccines, including whole-cell *Bordetella pertussis* vaccine and the influenza virus, rabies virus, and hepatitis A virus vaccines.

PURIFIED SUBUNIT VACCINES

A number of bacteria produce toxins that represent the major pathogenic components responsible for disease in infected humans. Examples include *Corynebacterium diphtheriae* and *Clostridium tetani*. Detoxified versions of these toxins are referred to as 'toxoids,' and represent the purified components of vaccines preventing diphtheria and tetanus, respectively. Toxoids have historically been produced by chemical inactivation of toxins, but more recently, genetic inactivation via targeted mutagenesis has been employed. The acellular pertussis vaccine is also a purified subunit vaccine composed of a defined set of protein constituents prepared from cultured *Bordetella pertussis*. The mechanism of immune protection conferred by purified subunit vaccines is the antibody response elicited by vaccination.

Antibodies directed against the capsular polysaccharides present on encapsulated bacteria also confer protective immunity in a number of important instances by inducing antibodies that exert opsonophagocytic

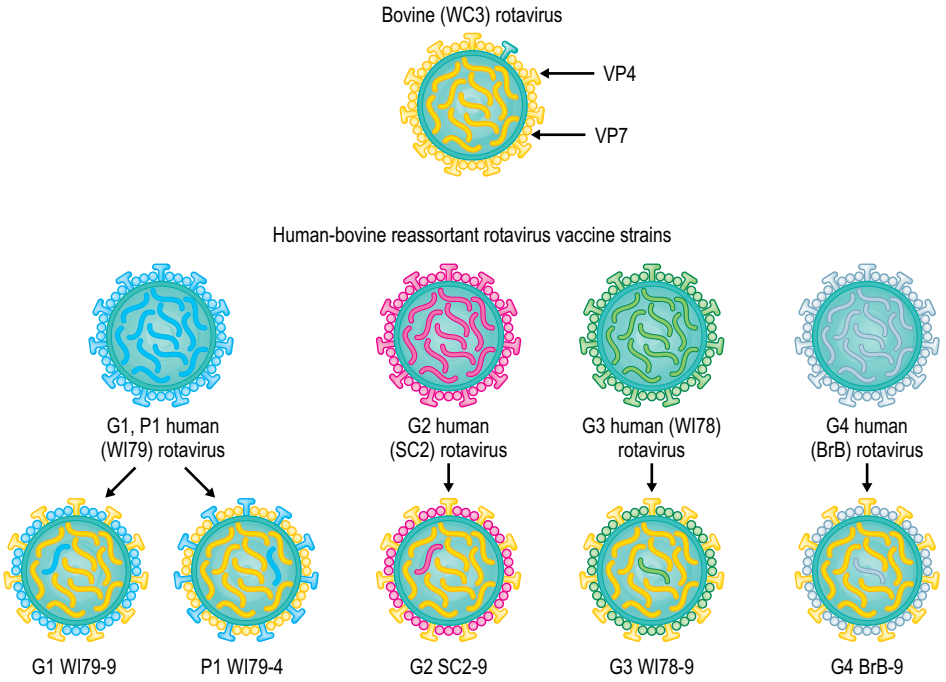

Fig. 92.4 New vaccine strategies: genetic reassortment approaches. Viruses with segmented genomes provide a new approach for the generation of attenuated vaccines via Mendelian genetic reassortment. If two such segmented viruses with different genetic characteristics are used to infect one cell, the progeny viruses from this mixed infection will carry a range of mixtures of the genes of the two parent viruses. Using either genetic or immunologic screening methods, reassorted viruses carrying the precise gene composition of interest can be selected. This approach has recently been employed to generate live attenuated vaccines against rotavirus and influenza virus. The strategy for generation of the pentavalent bovine–human reassortment rotavirus vaccine is shown above. Rotaviruses have a segmented double-stranded RNA genome comprising 11 independent RNA elements. The outer shell of the virus comprises two proteins VP4 and VP7 that are involved in cell binding and entry and that specify the viral serotype (P type for VP4 and G type for VP7). VP4 and VP7 also represent the targets of virus-neutralizing antibodies. The pentavalent bovine–human rotavirus vaccine was generated by a 'modified Jennerian' approach in which the bovine rotavirus WC3 (which is attenuated in humans as a result of host range restriction) serves as the gene donor for the backbone on to which gene segments encoding four common human rotavirus G types (G1–4) as well as one very common P type (P8) (derived from individual rotavirus isolates) were reassorted via a process of cell co-infection and subsequent selection of the recombinant viruses with the desired composition of bovine and human gene segments. An analogous genetic reassortment approach has also been used to generate live attenuated influenza vaccines. In this instance, three attenuated 'cold-adapted' viral strains (two A types and one type B) are used in co-infections in tissue culture with recent circulating wild-type influenza strains to derive vaccine strains that include the two relevant hemagglutinin (HA) and neuraminidase (NA)-encoding gene segments admixed with the six 'backbone' genes from the attenuated master donor virus for use in annual influenza vaccines.

effects (promoting phagocytosis of antibody-coated bacteria) and, in some instances, bactericidal effects.[17] Initial successful vaccine efforts against *Streptococcus pneumoniae* and *Neisseria meningitidis* utilized purified preparations of capsular polysaccharides. Although such purified polysaccharides can induce protective levels of antibody responses in adults, they are poorly immunogenic in children under 2 years of age (as a function of the relative immaturity of their immune systems). In addition, T-Independent antibody responses elicited by purified capsular polysaccharides are less durable than those that are produced in the presence of CD4 T-cell help. As a means of both augmenting antibody responses against polysaccharide antigens in young children and facilitating their persistence, the development of conjugate vaccines represented an important advance.[18] In this approach, purified polysaccharides are chemically conjugated to a carrier protein (such as diphtheria toxoid or an outer-membrane protein complex (OMPC) derived from *N. meningitidis*). The carrier protein augments CD4 T-cell helper responses to the polysaccharide antigens, and enables elicitation of durable protective antibody responses even in young children. Polysaccharide-conjugate vaccines have been produced that protect against *Haemophilus influenzae* b, *Streptococcus pneumoniae,* and *N. meningitidis* infections.

RECOMBINANT PROTEIN SUBUNIT VACCINES

The advent of recombinant DNA technologies provided a transformational event in the history of vaccine development. In addition to facilitating the identification and expression of pathogen-derived protective antigens, techniques were developed that enabled their large-scale manufacture as vaccines. Recombinant DNA technologies provided a new path to develop vaccines against pathogens, such as hepatitis B virus (HBV) or HPV, that could not be grown in culture. In addition, recombinant methods provided the potential to derive even safer versions of available vaccines.

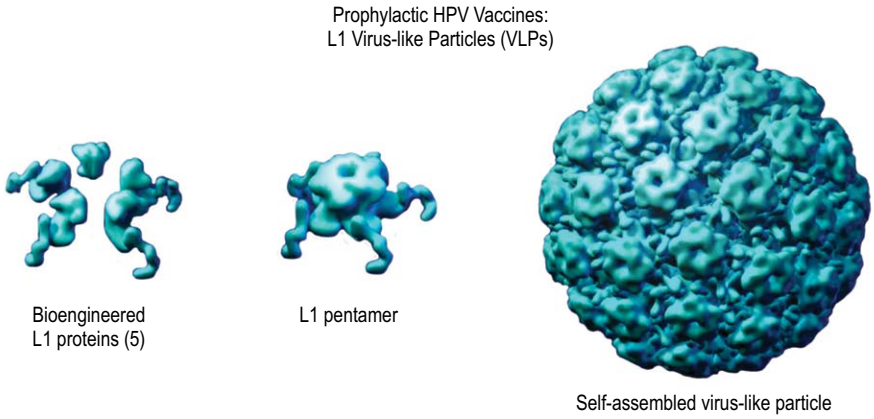

Fig. 92.5 New vaccine strategies: recombinant virus-like particle (VLP) approaches. In specific instances, VLPs can be produced via a process of self-assembly of individual viral capsid proteins produced by recombinant DNA methods in cell culture systems. This approach has a number of attractive aspects, including the ability to produce VLPs that accurately display conformationally correct epitopes recognized by neutralizing antibodies and the absence of pathogen-derived nucleic acids. In addition, recombinant VLPs have been employed to derive safe and effective vaccines for pathogens, such as hepatitis B virus (HBV) and human papillomavirus (HPV), that cannot be grown in culture (and are thus refractory to standard vaccine approaches of attenuation or inactivation). The generation of the VLPs that comprises newly developed HPV vaccines is shown. The HPV L1 proteins (which represent the major capsid protein and target of virus-neutralizing, protective antibodies), derived from HPV types of interest (e.g., types 16, 18, 6, and 11) are produced via recombinant methods. Under appropriate conditions, individual bioengineered L1 proteins first self-assemble into pentamers, and then into VLPs that are comprised of 72 pentamers and that are almost identical, both morphologically and antigenically, to infectious HPV virus particles. VLPs prepared from individual HPV types are then combined with specific adjuvants to prepare the final vaccine products.

The first recombinant vaccine developed, the recombinant hepatitis B surface antigen (HBsAg) prepared in yeast, was developed in hopes of avoiding safety concerns related to the plasma-derived HBsAg vaccine.[19] The knowledge that immune sera could provide protection by passive immunization of naïve hosts, and that purified inactivated plasma-derived HBsAg vaccine could elicit protective antibodies, laid the groundwork for development of this recombinant vaccine.[20] The recombinant vaccine, when combined with adjuvant (alum), elicits favorable immune responses, is highly efficacious and is well tolerated – all features that recombinant vaccines are now expected to deliver. The second recombinant vaccine developed targeted prevention of *Borrelia burgdorferi* infection (the cause of Lyme disease), and was based on a purified recombinant version of the OspA protein. This vaccine, although conferring some degree of efficacy, faced implementation challenges, and was not widely embraced. As a result, it was withdrawn from the market.

More recently, recombinant technology-derived purified subunit vaccines have been developed that consist of virus-like particles (VLPs) that self-assemble when the L1 protein of HPV is produced in isolation of other viral proteins (Fig. 92.5).[21] The L1 protein is the target of virus-neutralizing antibodies and vaccines consisting of a mixture of types 16 and 18 (the cause of ~70% of cases of cervical cancer) and 6 and 11 (the cause of ~90% of cases of genital warts) or of HPV types 16 and 18 alone have been shown to be highly efficacious and well tolerated.[22] Interestingly, HPV VLPs induce antibody responses that exceed those that follow natural HPV infections.[23]

In light of these successes, and the power and versatility of recombinant antigen production methods, a major proportion of new vaccine development efforts involves the use of protein subunit vaccines produced by recombinant technologies. Vaccines produced by this method are those that depend largely or exclusively on the induction of antibodies against individual or a selected subset of pathogen proteins. Because a number of proteins produced in isolation by recombinant methods have been observed to elicit lower immune responses than do natural infections or live attenuated vaccines, the development and use of adjuvants to optimize recombinant vaccine immunogenicity represent an important parallel area for future exploration.

VACCINE DEVELOPMENT AND EVALUATION

As a necessary prelude to clinical evaluation of candidate vaccines in humans, extensive preclinical research and development activities are undertaken to establish that the vaccine candidate has the desired properties. Toward this end, a number of key issues need to be addressed. First, animal studies must show that the vaccine candidate raises the desired type and magnitude of immune response against the infectious agent. Second, the vaccine needs to protect animals against death or disease in an appropriate challenge model, when feasible. Ideally, in the course of these studies, a specific type or level of immune response, referred to as a correlate of immune protection, can be identified. Third, the vaccine should be relatively free of serious discernible toxicities and side effects in animals when administered by the route intended for humans. Fourth, it is necessary to demonstrate that the vaccine can be produced in a consistent manner by a process that is consistent with the current good manufacturing practices (cGMP) process by which the first clinical trial materials will be produced (www.fda.gov/cber/gdlns/indcgmp.pdf).

KEY CONCEPTS

CONSIDERATIONS GUIDING VACCINE DEVELOPMENT

The development of new vaccines depends on the convergence of public health need, biological plausibility, and practical feasibility. Vaccine development programs are influenced by multiple considerations, including:

- What are the major unmet medical and public health needs today?
- What is known about the natural history and pathogenic mechanisms of the infection of interest?
- Is immunity to a given antigen associated with protection against disease following re-exposure in the context of natural infection?
- If natural immunity capable of preventing re-infection follows an initial infection with the pathogen, can a specific host immune effector mechanism (e.g., antibody, CD8 T-cell) be identified as the likely agent (or 'correlate') of immune protection? If so, can a threshold level of this specific immune correlate needed for protection from re-infection be defined?
- Can the pathogen be grown in culture? If so, does the pathogen cause such a life-threatening disease that an attenuated version of the virus would face an impossible barrier for demonstration of safety?
- Can a specific antigen (or antigens) be identified that represents the target of protective host immune responses?
- If the protective immune response is mediated by antibodies, can the target antigen (be it a protein or polysaccharide) be produced in scalable quantities in a form that mimics its native structure so that it can effectively elicit antibody responses that can block the key functional role(s) of the target molecule in the pathogen lifecycle or otherwise lead to the clearance of an incipient pathogen infection?
- Having chosen an antigen and presentation system, what is the best way to produce it on a large scale? Choices will be limited by the nature of the antigen and delivery system, but definition of an optimal system for producing the vaccine (prokaryotes like *Escherichia coli,* or diverse eukaryotic hosts including yeast, insect cells, plants, or cultured plant cells, mammalian cells) is a central consideration.
- What is the most effective way to present the antigens of the pathogen of interest to the immune system? Modern molecular biology and biochemistry have provided numerous options for vaccine immunogen presentation, including recombinant proteins (and recombinant virus-like particles (VLPs)), synthetic proteins, protein–polysaccharide conjugates, and gene delivery systems (recombinant viral vectors, or DNA vaccines)
- Is the antigen of interest sufficiently immunogenic on its own, or is augmentation of the desired immune response by conjugation to a specific carrier or addition of an adjuvant necessary to elicit a sufficient and sufficiently durable immune response in individuals in the target population for vaccination?
- What types of potential safety concerns can be anticipated for the vaccine in question?
- What is the attack rate of the infection in the general population? If the infection occurs relatively rarely in an overall population, can a subset of the population be identified that has a higher risk of infection so as to accelerate the achievement of statistically significant protection? Is this subset sufficiently similar to the rest of the population to enable extrapolation of the clinical results to the broader target population as a whole?
- What tests to evaluate vaccine immunogenicity will need to carried out on clinical samples obtained from participants in the clinical trials? Will measurement of antibody titers, T-cell responses, pathogen presence and quantity, pathogen serotype, and any other parameter peculiar to the disease in question represent the primary criteria for vaccine effect? Development and validation of theses tests represent an essential component for the feasibility and success of a vaccine clinical study

Even before preclinical studies are completed, vaccine developers typically begin an initial dialog with regulatory authorities (such as the Food and Drug Administration (FDA) or the European Medicines Agency (EMEA)) to set expectations about what will be necessary and sufficient for advancement to clinical studies in humans (www.fda.gov/cber/genetherapy/isct092506sh.pdf).

Phase I studies primarily focus on detailed assessment of the safety and tolerability of a vaccine, but evaluation of its immunogenicity is also frequently conducted. Generally, a phase I study includes fewer than 100 healthy volunteers divided unequally between those who receive vaccine or placebo (2 or 3 vaccinees per placebo recipient). Phase I studies typically employ escalating doses of the candidate vaccine, with a dose range progressively increasing in steps of three- to fivefold often being used. Blood samples are taken at prescribed intervals and analyzed for laboratory evidence of potential toxicity, as well as for evidence of vaccine-elicited immune responses. A phase I study is considered successful if it demonstrates that the candidate vaccine is well tolerated or identifies any immediate safety concerns that will need to be closely monitored in potential future clinical studies. Ideally, phase I studies also provide an initial indication of the optimal dose level and number of doses required.

A phase II study typically includes several hundred to a few thousand volunteers (randomized between vaccine and placebo) and can assume two general design types. Phase IIa studies provide additional safety data on a larger number of individuals of the intended age who receive the intended vaccine dose (who are more representative of the general population intended for vaccine use than the very healthy individuals included in the phase I study), as well as provide additional data on vaccine immunogenicity. Even larger phase IIb studies can provide additional data on vaccine safety and immunogenicity in subjects generally representative of those for whom the vaccine might be recommended, but importantly, also provide the first opportunity to address to answer the question, 'Does this vaccine work in humans?' The size of a phase IIb study needed to detect a signal of vaccine efficacy depends on the attack rate of the infection being targeted by the vaccine.

Phase II studies also present the first opportunity to identify a potential laboratory immunological correlate of protection from disease – if nature and prior experience have not already done so. In order to do so, the placebo recipients in the phase II trial must experience a sufficient number of cases of disease while vaccine recipients need to exhibit significant evidence of decreased risk of infection or disease. In addition, immunological measurements in the vaccinees need to capture the relevant protective immune responses (e.g., the type and level of antibody and/or cellular immune response that predict protection) and measure them with sufficient precision and reliability. If laboratory measurements of immunity correlate with vaccine protection, subsequent refinements of the vaccine, its adjuvant, its manufacturing process, or its regimen may be assessed by simple immunogenicity studies, rather than repeating efficacy studies. Once efficacy is established for a vaccine, it is very difficult to carry out a double-blinded, placebo-controlled efficacy study.

Vaccines that have been shown to be immunogenic and well tolerated in phase II studies can then advance to pivotal phase III studies required for vaccine licensure by regulatory authorities. Phase III studies are intended to expand further the safety database in a larger number of individuals (who are representative of the specific populations for which the vaccine will ultimately be used), establish definitive evidence of protective efficacy, and to establish clinical consistency of the vaccine made by the process run in the facility intended for licensure and commercialization (www.fda.gov/cber/genetherapy/isct092506jcr.htm). Typically, phase III studies include 10 000 or more subjects in a blinded, placebo-controlled design. This size trial allows the identification of less frequent safety events. It also provides an opportunity to capture data on health care utilization, cost, and impact of the vaccine on these parameters. As a new vaccine will ultimately be included in a vaccine program where multiple vaccines may be administered at the same time, it is also necessary to conduct concomitant-use studies. The developer of the new vaccine must show that the new vaccine does not impact on the immunogenicity of the existing vaccines, and that the existing vaccines do not impact on the immunogenicity of the new vaccine.

LICENSURE AND RECOMMENDATION OF VACCINES

In contrast to drugs, where licensure by the FDA is the primary determinant of how a new product is implemented in medical practice, vaccine use in the USA includes an additional process that evaluates how best to employ a new vaccine to optimize its implementation and public health impact. The US CDC has responsibility for making recommendations about the use of licensed vaccines, and it relies on its Advisory Committee on Immunization Practices (ACIP) for guidance. The ACIP considers several aspects in addition to a vaccine's safety and efficacy, including the anticipated cost-effectiveness and practical feasibility of potential alternative vaccine deployment strategies and consideration of how a new vaccine may be successfully implemented in clinical practice to achieve the greatest public health impact. Once the CDC has received, reviewed, and accepted the recommendation of the ACIP, the recommendation is published in its final official form in *Morbidity and Mortality Weekly Report* (MMWR; www.cdc.gov/nip/publications/acip-list.htm).

VACCINES FOR ROUTINE USE AND IN SPECIAL POPULATIONS

The recommended immunization schedule for children and adolescents (Fig. 92.1) is updated on an annual basis and can be accessed at www.cdc.gov/nip/recs/child-schedule.htm. The recommended adult immunization schedule (Fig. 92.2) is also updated on an annual basis and can be accessed at www.cdc.gov/nip/recs/adult-schedule.htm. The recommended adult immunization schedule includes information concerning use in special populations (such as health care workers and pregnant women) and individuals with specific conditions associated with altered or impaired immune function (such as individuals with congenital and acquired immunodeficiency syndromes, recipients of immunosuppressive therapies, malignancies, asplenia, liver disease, and renal disease). Readers are encouraged to check to ensure that they are following current recommendations.

Pregnancy registries currently exist for four vaccines in the USA. Health care professionals are encouraged to report exposures of pregnant women to the appropriate registry: HBV vaccine (800-670-6126), HPV vaccine (800-986-8999), meningococcal vaccine (800-822-2463), and varicella vaccine (800-986-8999).

VACCINE SAFETY

Unlike drugs that are utilized to treat individuals suffering from a given disease state, vaccines are administered to normal, healthy infants, adolescents, and adults. Consequently, standards for the safety and tolerability of vaccines are set at a very high level. When developing a new vaccine, a graded process of clinical studies is employed that involves increasingly larger numbers of volunteers and that typically progresses from individuals who are selected to be free of any identifiable health problems to those who are selected to be representative of the overall population for whom the vaccine is being developed. If phase I studies reveal no evidence of safety concerns and the desired evidence of immunogenicity, a major focus of the series of larger randomized double-blind, phase II placebo-controlled studies that are then conducted is to explore the safety and tolerability of a vaccine in increasingly vulnerable populations (such as those who may have identified pre-existing health problems or asymptomatic abnormalities detected on screening laboratory studies).

Reflecting the importance of documenting the safety of a new vaccine, phase III studies to assess the safety and efficacy of a new vaccine now typically involve large numbers of volunteers. Indeed, as a result of needing to provide evidence for safety, it is now common to have the size of the phase III trial be significantly larger than would be necessary to document vaccine efficacy. The ability of a study to identify an increased risk of any given adverse event with sufficient statistical power is directly related to the size of the population in the study. As a general rule, a study of 300–400 subjects is needed to measure the risk of an event that happens in one out of 100 individuals. For one in 1000, 3000–4000 subjects are needed. Even in studies of this size, very rare events may not be identified, and if a specific safety concern exists substantially larger trials may be needed.

The recent experience with the development of rotavirus vaccines provides an illustrative example of the importance placed on documenting vaccine safety.[24] Rotavirus is an important cause of serious gastroenteritis

in infants and young children, and the associated diarrhea and vomiting can lead to life-threatening dehydration. In developing countries where health care resources and effective rehydration options are limited, over 600 000 infants die of rotavirus gastroenteritis each year.[25] Given the global importance of rotavirus gastroenteritis, the first licensure of an orally administered rotavirus vaccine in 1998 was a very welcome advance. However, as the vaccine entered routine pediatric practice, it was recognized that a low, but increased incidence of intestinal intussusception was seen after the first and second doses (with about one case of intussusception seen per 10 000 vaccinees.)[26] Upon recognition of this association, the vaccine was withdrawn from the market.[27]

With the evident public health need for a safe and effective rotavirus vaccine, it was hoped that alternative rotavirus vaccines then in development (both oral vaccines based either on a combination of bovine–human reassortant viruses (Fig. 92.4) or an attenuated human rotavirus strain) might differ from the first licensed rotavirus vaccine and not result in an increased rate of intussusception. However, to demonstrate that these alternative rotavirus vaccines were safe, and that an increased risk of intussusception was not inherent to rotavirus vaccines as a class, very large-scale safety studies were required. Toward this end, the safety of each of these vaccines was evaluated in studies involving about 70 000 infants – just to evaluate whether the rate of intussusception in vaccinees was discernibly increased compared to the normal background rates seen in the placebo recipients.[10,11] Fortunately, both vaccines were found to be well tolerated and no increase in intussusception was observed in vaccine as compared to placebo recipients. In light of the documented efficacy of these vaccines determined in earlier and significantly smaller phase III trials, both have now been licensed in a number of countries. However, even with the large phase III studies conducted for these newer rotavirus vaccines, they will still be studied in large postlicensure active surveillance safety studies and closely monitored in active and passive vaccine safety surveillance systems (see below).

Following vaccine licensure, safety is tracked via a number of means, including both active and passive surveillance studies of adverse events. Active surveillance includes phase IV postmarketing studies of vaccine safety in larger populations in real-world use. Formal postmarketing studies can include tens of thousands of individuals or more.

An alternative type of postmarketing safety study is carried out by the US FDA and the CDC within the context of the Vaccine Adverse Event Reporting System (VAERS) database (www.vaers.hhs.gov or by telephone: 800-822-7967). The VAERS database accepts spontaneous reports of adverse experiences from health care providers, patients, parents, vaccine manufacturers, and other sources.[28] The best use of the VAERS database is to identify signals in a population that may appear following the introduction of a new vaccine.

A newer vaccine safety surveillance system, known as the Vaccine Safety Database (VSD), has been developed by the CDC in cooperation with seven large health maintenance organizations (HMOs) around the USA.[29] The VSD contains the complete medical records of all the members from the participating HMOs, and the information used to populate the database is entered by health care professionals using relatively consistent terminology, improving the quality, uniformity, and usefulness of the data. Particularly important is that the VSD construct allows comprehensive epidemiological analyses to determine if the incidence rate of a specific adverse event is higher among vaccinees than nonvaccinees. In addition to VAERS and the VSD, the CDC has also created a Clinical Immunization Safety Assessment Network that reviews patterns of clinical syndromes that may follow vaccination.

While the safety profile of a vaccine can be relatively well defined through the efforts described above, confidence in vaccination programs has often been challenged by public perceptions, either real or unsubstantiated, about vaccine safety. In some instances, specific vaccines have been associated with increased incidence of a specific adverse experience, such as the association between the first-generation rotavirus vaccine and an increased risk of intussusception following vaccination. However, a number of other safety concerns that have emerged are not supported by scientific evidence. An example of this can be found in the case of concerns about the association of whole-cell pertussis vaccines with permanent brain damage – concerns that were later shown to be unfounded. Nevertheless, public concerns about the safety of the whole-cell pertussis vaccine resulted in decreased levels of pertussis vaccination coverage that were soon followed by epidemics of whooping cough in the UK and Japan.[30] Another example is the allegation that certain vaccines, such as the combination measles, mumps, rubella (MMR) vaccine, are associated with autism. Highlighting how perceptions of temporal association can give rise to public concerns, MMR vaccines are generally given around 1 year of age, and autism is generally diagnosed in the second year of life. Although the alleged causal association between MMR and autism has been refuted by thorough scientific analyses, reports in the popular media in the UK resulted in a dramatic drop in vaccination rates, followed by an increased rate of new infections.[31,32]

VACCINES NOT YET AVAILABLE

Although an impressive armamentarium of vaccines is now available, safe and effective vaccines have yet to be developed for a number of very important infectious diseases. The reasons underlying the lack of effective vaccines for an array of important pathogens include biological considerations, safety concerns, and practical constraints. Of these, the biological considerations are often the most important barrier. As discussed above, vaccines have been successfully developed for pathogens whose natural infections give rise to natural immunity wherein the infected host (at least those who survive initial infection) is no longer susceptible to re-infection (such as measles, yellow fever virus, or smallpox) or who experiences significantly less severe clinical sequelae upon re-infection (such as rotavirus). In instances where natural immunity follows natural infection, not only is a precedent for immune protection established, but the nature of protective host responses can be studied, providing a correlate of protection to guide vaccine development efforts. However, for many of the pathogens for which vaccines remain elusive, natural immunity does not follow natural infection. In the absence of natural immunity, not only is a precedent for successful immune containment lacking, but no potential correlates of protection are available to inform vaccine development. In some instances where natural immunity does not follow natural infection, persistent infections are established and maintained by active virus replication that cannot be controlled or cleared by host immune responses (such as HIV and hepatitis C).

Alternatively, other pathogens are able to persist in the host through establishment, via diverse mechanisms, of latent infections that are resistant to host immune clearance (such as tuberculosis or herpes viruses (such as herpes simplex virus (HSV) or Epstein–Barr virus (EBV))). In other instances, even when the host is cleared of an infection via drug

treatment, the host remains susceptible to re-infection and disease in the future (such as malaria). Although different pathogens have evolved diverse strategies for evasion of host immune responses – ranging from manifestation of tremendous genetic diversity and propensity for immune escape; to sequestration of critical structural domains that might be susceptible to antibody neutralization; to the utilization of specific mechanisms to evade host innate and adaptive immune effectors – the common end result is frustration of vaccine development.

While failure of host clearance of an infection is a common theme underlying the lack of vaccines, additional obstacles to vaccine development include other immunologically related considerations as well as both practical and safety considerations. Examples of immunologically related obstacles include instances where prior exposure to a given pathogen predisposes the host to more severe disease manifestations upon re-infection (as has been proposed in the case of dengue virus) or where earlier vaccine development efforts inadvertently lead to severe adverse events following infection with the targeted pathogen (such as respiratory syncytial virus (RSV)). In each of these cases, the adverse events that follow a secondary immune exposure are believed to be the result of immunopathologic responses that result from the nature of the immune response elicited by the initial exposure to pathogen-derived antigens (by either infection or vaccination). Given that the mechanisms underlying these immunopathologic processes are incompletely understood, the development of vaccines that are highly immunogenic but not similarly inclined to elicit immune-mediated adverse consequences represents a substantial challenge (especially given the very high expectations for vaccine safety). An additional immunologically related challenge relates to the observation that certain organisms encode antigens that resemble constituents of the human host. For example, in the case of *Neisseria meningitidis* group B, the bacterial polysaccharide resembles those found on certain human cell lineages, thus raising concerns about whether polysaccharide-based vaccines successfully developed for group B *N. meningitidis* might yield undesirable autoimmune responses.[33]

An additional distinct, but important, practical barrier to new vaccine development relates to the prevention of diseases that are threats to pregnant women or their offspring (where immunization of the pregnant woman might be able to protect the neonate). Although a number of inactivated vaccines are either routinely recommended for use in pregnant women (e.g., inactivated influenza vaccine) or can be used in pregnant women for pre- or postexposure prophylaxis for those at risk of infection (e.g., inactivated hepatitis A vaccine and recombinant HBsAg vaccine), the development of new vaccines specifically for use in pregnant women or the study of new vaccines in pregnant women has been impeded by concerns arising from potential litigation that might follow the appearance of a congenital abnormality in a child born to a mother who was vaccinated while pregnant.[34] Given the 2–3% prevalence of congenital abnormalities, the practical difficulties in proving the safety of a new vaccine specifically administered to pregnant women, and the current litigious environment surrounding vaccines, the development of new vaccines to address important infections of pregnant women and their neonates (e.g., group B streptococcus: GBS) faces significant challenges.

There remain a number of important infectious diseases for which no effective preventive vaccines exist. Below, we list the major 'missing' vaccines, comment on why they are not yet available, and highlight the major approaches currently being explored to develop them.

HUMAN IMMUNODEFICIENCY VIRUS (CHAPTER 37)

At the end of 2006, an estimated 40 million people were living with HIV infection, and in the preceding year approximately 4.5 million people became newly infected, and approximately 3 million individuals died of AIDS. As the most promising biomedical intervention to contain the AIDS pandemic, the development of an HIV vaccine is a top global health priority. Yet, HIV infection represents a vexing challenge to vaccine development.[35,36] HIV infection does not result in clearance of the virus due to a host immune response. Following infection of target cells, the genome of HIV – a retrovirus – is transcribed into a DNA copy via the action of reverse transcriptase. The newly formed DNA copy of the HIV genome then integrates into the host cell chromosomes (referred to as a provirus) as a requisite step in the viral lifecycle. Once integrated into the chromosome of an infected cell, the HIV provirus can alternatively be actively transcribed, leading to the synthesis of viral mRNAs and subsequently to production of new virus particles, or it can remain in a transcriptionally silent, functionally latent state in a small percentage of infected cells. As infected cells harboring latent HIV proviruses do not produce HIV protein antigens, they cannot be recognized by host antiviral immune responses and can thereby persist undetected. Upon subsequent activation of latently infected cells at some later time, viral RNA transcription can be coincidently activated leading to production of progeny virions.

As HIV targets activated CD4 T cells for infection and consequent depletion, the host's ability to mount both HIV-specific and non-HIV-specific immune responses is progressively impaired. The ability of the host to clear HIV infection is further complicated by the extensive genetic diversity of virus populations that emerge, and progressively diverge, within infected individuals as a function of a replicative cycle that is accomplished by the inherently error-prone reverse transcriptase and the numerous cycles of replication that occur in infected individuals. As a result of these influences, genetically diverse populations of HIV variants are established in infected persons that facilitate the outgrowth of genetic variants that can escape from selective pressures – be they effective host cellular or humoral immune responses, or the inhibitory effects of antiretroviral drugs.[37] An extraordinary degree of genetic diversity is also manifest in the HIV variants seen in different individuals and in different geographic regions. As successful vaccines for other infectious agents have historically had to protect against pathogens exhibiting only limited genetic diversity, HIV represents an unprecedented challenge.

As many successful vaccines protecting against viral infections are predicated on the induction of neutralizing antibody responses against the viral surface proteins that mediate attachment to and entry into target cells, significant efforts have focused on the potential of the HIV surface envelope (Env) glycoprotein, Gp120, to elicit infection-neutralizing antibodies.[38] Unfortunately, HIV gp120 is highly resistant to the action of antibodies by virtue of its heavy glycosylation and its native conformation that shields functionally critical structural domains from antibody binding. As a result, candidate gp120-based vaccines have failed to elicit meaningful levels of neutralizing antibodies in immunized human volunteers and have not protected from HIV infection in two large phase III studies.

Given the inability, to date, of candidate HIV Env-based vaccines to elicit appreciable levels of neutralizing antibodies, current vaccine strategies are largely focused on the induction of CD8 cytotoxic T-cell responses against the more constrained and conserved antigens, such as

gag, pol, and *nef*. It has been hypothesized that induction of high levels of HIV-specific CD8 T-cell responses prior to infection may not prevent infection, but may enable infected individuals to control virus replication better. Should this hypothesis be valid, individuals immunized with such vaccines may exhibit lower levels of ongoing HIV replication, progress to AIDS more slowly, and potentially be less likely to transmit HIV infection to others. Much of this work involves vectored gene delivery systems (such as adenoviral vectors, described below). However, the recently announced results of a phase IIb 'test of concept study' [39] failed to demonstrate a beneficial effect on either prevention of infection or reduction of viral load among volunteers who received the vaccine despite the induction of appreciable levels of HIV-specific CTL responses by the recombinant adenovirus-based vaccine employed. While this study result does not, in and of itself, refute the 'CTL hypothesis', it represents a significant disappointment for the AIDS vaccine research effort, and raises important questions about the ability of vaccine-elicited cell mediated immune responses to favorably alter the outcome of HIV infection.[39a] There are also efforts under way to utilize the recently solved three-dimensional structure of the HIV Env glycoprotein to guide the derivation of non-native structures that might serve as better immunogens to elicit broadly cross-reactive neutralizing antibodies. In addition, relatively conserved and functionally essential sequences of the extracellular domain of the HIV transmembrane Env protein, gp41, are being explored as immunogens to elicit broadly neutralizing antibodies.

MALARIA (CHAPTER 29)

Malaria is the world's most common vector-borne disease – estimated to cause approximately 500 million clinical cases and 2 million deaths annually.[40, 41] The disease hits hardest in Africa, and is especially severe in children under 5 years of age. In addition to direct morbidity and mortality, malaria is responsible for debilitating illness with enormous social and economic consequences. Of the four malaria-associated protozoal species, *Plasmodium flaciparum* and *P. vivax* represent the two major agents. These parasites have a three-stage lifecycle taking place both within the mosquito, and in the liver and blood of the infected host, and each cycle is largely distinct from the others from an immunological perspective. As a result of the multiple strategies for evasion of host immune response that the parasite has evolved, parasite replication proceeds at high levels despite active host immune responses.[42, 43] Either as a result of these specific immune evasion strategies or the inability of the infected human host to mount immune responses that clear the parasite, prior infection does not protect an individual from repeated subsequent infections. Although the severity of disease is often attenuated following repeated infection, the mechanism of disease modulation is incompletely understood, and the limited relative immunity engendered by prior infection is easily lost if an individual leaves a malaria-endemic region. As such, the limited impact and duration of host immune responses to malaria parasites suggest that any successful vaccine strategy will need to do far better than natural immune responses – a high bar for efforts to develop an effective vaccine.

Roughly two dozen antigens have been cloned and tested as potential vaccine immunogens, and with a few exceptions the results have been disappointing.[44] One antigen, the circumsporozoite antigen, presented as a fusion with HBsAg (RTS,S), has shown modest promise in human studies.[45] This vaccine is now undergoing larger-scale clinical efficacy testing to determine if the magnitude of protection would justify large-scale implementation efforts. Should 'proof of concept' be supported in these studies, but the absolute magnitude of efficacy be insufficient, future efforts will likely focus on the identification of an appropriate adjuvant to improve the magnitude and duration of immune responses. Alternative approaches include immunization with irradiated or genetically attenuated sporozoites.[46] definition of novel antigens expressed at specific stages of the parasite lifecycle, and evaluation of combinations of multiple parasite antigens.

TUBERCULOSIS

Mycobacterium tuberculosis is an intracellular mycobacterial pathogen that represents one of the world's most common and most serious infectious diseases.[47] Over 2 billion people are believed to harbor latent *M. tuberculosis* infections, and approximately 8 million active cases of tuberculosis and over 2 million deaths occur each year. Furthermore, the interface of HIV infection and its attendant immune system damage both increases the severity of *M. tuberculosis* infection and increases the infectiousness of infected individuals. The emergence and dissemination of *M. tuberculosis* isolates that are resistant to multiple antimicrobial drugs represent a growing public health threat. However, while the need for a vaccine to prevent tuberculosis is clear, a significant number of challenges face vaccine development efforts.[48] Most individuals infected with *M. tuberculosis* can control the acute phase of mycobacterial replication, and mount vigorous innate and adaptive immune responses to the infection. However, the infection is often not cleared by the host's immune response, and the mycobacteria are able to persist and multiply within vacuoles inside macrophages. Long-term latency is established in fibrotic cysts in the lung. The recrudescence and dissemination of *M. tuberculosis* occur at a later time in a number of infected individuals, likely as a result of waning host immune control. Although the ability of *M. tuberculosis* to persist despite active innate and adaptive immune responses represents a major challenge to vaccine development, the fact that most individuals can contain (if not clear) *M. tuberculosis* infection suggests that a vaccine that can alter the course of the natural infection by limiting early dissemination and decreasing the risk of later recrudescence could provide major public health benefits.

Efforts to develop a vaccine against tuberculosis date back many decades. Bacille Calmette-Guérin (better known as BCG), based on *Mycobacterium bovis*, was first introduced in 1921.[49] Currently, BCG is provided as a component of the routine Expanded Programme for Immunization (EPI) schedule and is administered to a significant majority of the world's children. Although some protective efficacy (50–80%) has been reported against miliary infection and *M. tuberculosis* meningitis in children, conflicting results have been obtained in different studies regarding the ability of BCG to protect against pulmonary tuberculosis in adults. One explanation for the overall limited efficacy of BCG emerges from formal genome sequencing studies that have disclosed significant differences between *M. tuberculosis* and of the vaccine strain of BCG. The variability in the results of BCG efficacy studies in different populations and geographies may derive from variations in the geographic prevalence of cross-reactive mycobacterial species (that may themselves confer partial protection), or the fact that BCG vaccines used throughout the world do not represent a homogenous preparation – with the root strain of BCG having been widely distributed and passaged extensively under diverse conditions.

Vaccine efforts against tuberculosis have primarily focused on the evaluation of specific mycobacterial antigens (e.g., ESAT6, Ag85, and HSP60) that have been tested as vaccines in animal models with variable success.[50, 51] Some of these strategies are now being advanced into human clinical

trials. An alternative strategy is based on improving the performance of the BCG vaccine by insertion of genes encoding specific potential protective antigens that it normally lacks. In addition, the development of auxotrophic mutants of *M. tuberculosis* is being explored as a potential immunogenic and specifically attenuated live vaccine. The determination of the sequence of the *M. tuberculosis* genome nearly a decade ago helped identify numerous previously unknown gene products, and increased the repertoire of antigens to be evaluated for their ability to induce protective immune responses.[52] The pathogen sequence is also being used to elucidate virulence determinants and thereby help guide efforts to attenuate *M. tuberculosis* rationally.

RESPIRATORY SYNCYTIAL VIRUS AND PARAINFLUENZA VIRUS (PIV)

Together with influenza virus, RSV and PIV account for a substantial majority of pediatric upper respiratory illness and consequent acute otitis media. A variety of influenza vaccines are licensed for pediatric use, but vaccines to prevent infection with the paromyxoviruses RSV and PIV remain elusive. A significant impediment to vaccine development for RSV and PIV traces back to unanticipated untoward results obtained in clinical studies of inactivated RSV vaccines in the early 1960s.[53] These early-generation RSV vaccines – based on cultured virus that had been inactivated with formalin – raised a potent antibody response in immunized children. However, on subsequent natural exposure to RSV, vaccine recipients exhibited more frequent and significantly more severe lower respiratory tract RSV infections than did unimmunized children. As a similar phenomenon was also seen with a formalin-inactivated measles vaccine in the same era, a common immunopathologic mechanism may be operative.[54] While the mechanism of exacerbation of RSV disease by the early inactivated vaccines is incompletely understood, it has been suggested that chemical inactivation of RSV and measles resulted in modification of a critical neutralizing structure on the surfaces of these viruses, thereby limiting the induction of the most potent neutralizing antibodies and favoring nonneutralizing and potentially immunopathologic antibody responses. (Passive protection against RSV is available for premature infants in the form of monoclonal antibodies that target the RSV F protein (one of the viral envelope glycoproteins); certain anti-RSV antibody responses can clearly mediate protective as opposed to deleterious effects.[46]) Alternatively, or in addition, it has been proposed that inactivated RSV vaccines may have preferentially induced a Th2-type immune response when a Th1-type response may be needed to effect protection of the lower respiratory tract from RSV infection and damage.

While excellent live attenuated measles vaccines have been developed, RSV and PIV have so far resisted the approach used for measles and mumps (these are all members of the Paramyxoviridae family of viruses). Based on the successful precedent provided by the live attenuated measles vaccine, an attenuated or reverse genetics-engineered RSV is considered the most promising approach. However, stable attenuation of RSV has been difficult to achieve and vaccine safety concerns result in their cautious advancement through clinical evaluation.[55]

NEISSERIA MENINGITIDIS GROUP B

Effective vaccines for meningococcus types A, C, Y, and W135 are available as straight capsular polysaccharides and as conjugated polysaccharides.[56] The group B polysaccharide shares chemical similarity with a shorter sugar found on the surface of neuronal tissue.[57] While it is possible to make highly immunogenic conjugates with the group B polysaccharide, theoretical concerns about cross-reactivity with self antigens has impeded the development of this type of vaccine. Current work centers on a handful of relatively well-conserved surface proteins of meningococcus.

GROUP B STREPTOCOCCUS

GBS is a common component of the flora of the female genital tract, and transfer to the neonate is the cause of severe infections that are fatal or have serious sequelae.[58] Short-course intrapartum antibiotics are recommended for culture-positive women, and this approach has cut the incidence of neonatal infections by about two-thirds, thus reducing somewhat the urgency of vaccine development. However, short-course antibiotics could ultimately drive the emergence of antibiotic-resistant GBS. Candidate vaccines have been shown to elicit a protective response.[34] However, aside from a reduced market, the main impediment to development of a GBS vaccine is concern over vaccination of pregnant women or women of childbearing age. Any birth defect might be attributed to the vaccine, and in a litiginous society, this would be problematic for a vaccine producer.

HEPATITIS C VIRUS (HCV)

Prior to the advent of effective polymerase chain reaction methods for screening blood donations, HCV was a significant cause of transfusion-related hepatitis. Currently, transmission of HCV among the normal population is quite low; transmission among injection drug users remains high. HCV is another pathogen where infection does not typically result in an immune response that clears the infection. However, a minority of HCV patients do spontaneously clear their infection, suggesting that an appropriate immune response could do the job. Current vaccine work is concentrated on vectored gene delivery vaccines, primarily adenoviruses, intended to raise antiviral cytotoxic T-cell responses.[59]

HERPES SIMPLEX VIRUS

With the exception of the live attenuated varicella-zoster virus (VZV) vaccine used for the primary prevention of chickenpox and reactivation of latent VZV infections (the cause of shingles and postherpetic neuralgia in older individuals), there are no other vaccines available for use in humans to prevent infection with members of the herpes virus family.[60] HSV types 1 and 2 cause recurrent vesicular eruptions "above or below the belt," respectively. Like other herpes viruses, HSV infections are not cleared by the immune system and the virus can persist, remaining in a latent state that is functionally inaccessible to immune recognition and clearance. In addition, like other herpes viruses, HSV encodes a number of gene products that promote evasion of host immune responses. Recent attempts to make HSV2 vaccines have used virus glycoproteins produced by recombinant DNA methods. A recent clinical efficacy trial of this vaccine approach showed partial protection of women, but not men, who were seronegative for HSV1.[61] The reasons for this curious result are not clear, but efforts to develop this type of vaccine continue. In addition, a number of preclinical studies are exploring the ability of cell-mediated immune responses to HSV antigens induced by recombinant vaccine vectors (e.g., adenoviruses: see Novel vaccine vectors, below) to prevent or ameliorate HSV infections. Genetically engineered attenuated HSV variants have also been studied in experimental animal models. It is not clear when these new strategies may advance to clinical evaluation in humans.

KEY CONCEPTS

CONTEMPORARY OPPORTUNITIES AND CHALLENGES IN VACCINE DEVELOPMENT

The processes of vaccine development have changed significantly in recent years – a process facilitated by substantial improvements in understanding of human immune system function, as well as the advent of powerful new technologies for vaccine development. As a result of these advances, vaccine development is now commonly pursued in a hypothesis-driven manner and is a far less empiric pursuit than in the past. However, at the same time, the infectious diseases for which no effective vaccines currently exist represent more challenging targets than those diseases that have yielded to vaccine development efforts in the past. Furthermore, global changes that influence the emergence and rate of spread of infectious diseases place unprecedented challenges on the productivity and pace of new vaccine development efforts.

Current opportunities

- Improved understanding of human immunology (including the biology of innate immune system function, antigen presentation, and the generation and maintenance of T- and B-cell memory)
- Improved technologies to measure human cellular and humoral immune responses
- The advent of genomic and proteomic technologies for new antigen discovery
- The wealth of recombinant DNA methodologies that enable the isolation and characterization of protective antigens from diverse pathogens (including those that may not be successfully propagated in culture)
- The development of recombinant and synthetic approaches for the large-scale production of precisely defined vaccine antigens (including the ability to produce immunogens that accurately recapitulate the conformational structure of native antigens, or that, alternatively, alter them so that they serve as more effective immunogens in eliciting desired immune responses)
- The emergence of new mechanism-based vaccine adjuvants to enhance the immunogenicity of vaccine antigens

Current challenges

- The need to develop vaccines for infections where natural immunity does not often or ever develop following natural infection (e.g., human immunodeficiency virus (HIV), malaria, hepatitis C)
- The need to develop vaccines that protect against genetically diverse pathogen variants with a limited number of vaccine immunogens (e.g., HIV, malaria, and influenza)
- The need to develop vaccines for infections where concerns exist about vaccine elicitation of potentially autoimmune (*Neisseria meningiditis* group B) or immunopathologic (e.g., respiratory syncytial virus) responses by vaccination
- The challenge of responding rapidly and effectively, with powerful new technologies, to newly emerging infections – including those that haven't been seen in humans before (e.g., severe acute respiratory syndrome (SARs)) or for which novel antigenic variants are anticipated but cannot be predicted (e.g., pandemic influenza)
- Maximizing the value of innovative new approaches while ensuring the safety of new vaccines so derived

CYTOMEGALOVIRUS (CMV)

Another herpes virus, CMV is a very common infection in humans, with 50–80% of individuals being infected by adulthood. CMV is a cause of severe infections in neonates, causing debilitating neurological sequelae. Following initial infection, CMV persists in infected humans, despite the fact that anti-CMV antibodies are present and that a very sizeable proportion of the overall host CD4 and CD8 immune responses are specific for CMV antigens. Ongoing virus persistence and replication in the face of active host immune responses are likely explained by CMV's sophisticated repertoire of host immune evasion functions (including those that inhibit antigen presentation mechanisms and immune effector responses). For these reasons, to be successful, vaccine development efforts will need to elicit immune responses that are significantly more effective than the quantitatively impressive, but functionally limited, immune responses that are generated in the course of natural CMV infections. Live attenuated vaccines have been investigated sporadically since the 1970s.[62] An attenuated strain, the Towne strain, showed some effect, but was judged to be insufficiently immunogenic. Hybrids of the attenuated Towne strain and the virulent Toledo strain remain in development. Recent work has included recombinant DNA (rDNA)-derived proteins (via either DNA vaccine approaches or recombinant viral vectors, such as attenuated poxviral vectors).[63,64]

EPSTEIN–BARR VIRUS

EBV is a herpes virus that represents the causative agent of infectious mononucleosis and is widespread among the human population. In concert with incompletely understood environmental (and perhaps additional host) factors, EBV is also etiologically associated with Burkitt's lymphoma. The ability of EBV to establish persistent infections in humans (along with latent infections at the cellular level) despite readily detectable antiviral immune responses suggests that, like other herpes viruses, the development of effective EBV vaccine will likely be challenging. EBV vaccines have been in development since the 1980s with the coat protein, gp220/350, as the most common vaccine antigen studied.[65]

DENGUE FEVER VIRUS

Dengue fever virus is a mosquito-borne flavivirus (the virus family that includes Japanese encephalitis virus and yellow fever virus – for which successful vaccines exist). Dengue virus is endemic in a substantial portion of tropical and subtropical areas and causes febrile disease as well as hemorrhagic fever. There are four distinct serotypes of dengue fever virus. Prior infection with one serotype has been implicated in predisposing for more severe disease following infection with a second dengue fever virus serotype, although the evidence supporting this concept has been questioned and the underlying pathogenic mechanisms are incompletely understood.[66]

One hypothesis proposes that antibodies against the initial infecting serotype bind to the surface of virus particles of the novel infecting serotype, but do not neutralize the infection. In a process referred to as "immune enhancement" of infection, still infectious complexes of antibody virus particles are then envisioned to be preferentially taken up by cells of the reticuloendothelial system that represent primary target cells for virus replication. Although the veracity of this hypothesis in not established, it does present certain theoretical concerns about what type of antibody responses will need to be induced by vaccines to exert beneficial rather than detrimental effects. The general belief is that a vaccine providing equivalent immunity against all four serotypes will be required. Vaccines based on inactivated virus, engineered chimeric viruses based on the yellow fever virus vaccine platform, engineered deletion mutant viruses, and rDNA-derived proteins are in various stages of development.[67]

NEW ANTIGEN DISCOVERY METHODS

Historically, vaccine antigens were not discovered in the literal sense. Rather, whole organisms were inactivated by either heat or chemistry or organisms were attenuated by forcing growth in nonphysiological conditions. The entire antigenic repertoire of the organism was delivered to the immune system. The isolation of tetanus, diphtheria, and pertussis toxins, along with chemical detoxification schemes, allowed the production of more refined vaccines. The isolation and purification of polysaccharide capsules from a range of important bacterial pathogens enabled the development of additional vaccines.

With the advent of molecular biology in the late 1970s, a new set of tools allowed a more directed approach for the discovery of pathogen virulence factors, vaccine antigen discovery, and vaccine development. The tools of molecular biology enabled for the first time the development of vaccines against pathogens that could not be propagated in culture, including the successful development of recombinant HBV and HPV vaccines. Development of these vaccines was enabled by clinical and animal model studies showing that antibodies directed against a specific viral target antigen (e.g., the HBV surface antigen or the HPV L1 protein) were implicated in protection. In addition, molecular biologic approaches enabled the derivation of fully recombinant vaccine antigens (such as those developed using a limited set of defined antigens of *Bordetella pertussis*), including genetically modified versions of bacterial toxins that maintain their proper antigenic structures but are no longer toxic. However, for many of the pathogens for which vaccines do not currently exist, application of these recombinant DNA technology-enabled strategies are insufficient due to incomplete understanding of the pathogen antigens that would elicit a protective host immune response. As such, the development of additional techniques to discover protective antigens was needed. Fortunately, several important technological advances that facilitate discovery of previously unknown protective antigens from even very complex microorganisms have opened a new era in vaccine development.

The earliest rDNA technology-enabled methods of antigen discovery involved the expression of individual pathogen-derived gene products (or fragments thereof) in bacterial hosts (typically *Escherichia coli*) using rDNA expression vectors. Here, the genome of a pathogen is broken up, and the fragments are inserted into a plasmid or a viral vector, typically a lambda bacteriophage.[68] Colonies, or plaques, are spread on a membrane, allowed to grow, and hopefully express the cloned gene fragments. The ability of these recombinant gene products (now isolated in individual colonies) to react with antibodies present in the serum of individuals who had recovered from infection with that pathogen could then be directly assessed. Antibodies present in the immune sera are assessed for their ability to identify antigens by immunochemical reactivity. In this way, the entire genome of a given pathogen could be scanned for potential immunoreactivity.[69] Such reactivity would both indicate the *in vivo* expression of that gene product, as well as document its antigenicity. However, additional studies are needed to demonstrate whether antibody responses against a newly defined antigen have any protective potential. To document the ability of an antigen to elicit protective immune responses, it is necessary to immunize an experimental animal (most commonly, mice) and then, following experimental pathogen challenge, evaluate infection outcomes in immunized versus nonimmunized animals. As this approach has most often been used to identify antigens recognized by host humoral responses, sera from animals immunized with a candidate antigen can then be transferred to a naïve host to provide evidence that the antibody response to the antigen represents the relevant agent of immune protection.

More recently, as DNA sequencing became more efficient and scaleable, determining the entire sequence of the genomes of viruses, bacteria, and parasites has become routine,[70, 71] allowing identification of previously unknown genes (and predicted gene products) that can be evaluated as vaccine immunogens. Scanning the entire pathogen genome via specific computer analysis programs, genes that exhibit specific characteristics can be identified (e.g., predicted expression on the cell surface by virtue of possession of a leader sequence for secretion or membrane anchor sequences).[72] In addition, the relative conservation of the gene within the pathogen population can be determined by assessment of gene sequences from multiple distinct isolates. Once potential vaccine antigens are identified, each candidate gene is expressed in an appropriate rDNA system, and the protein product is tested in an animal model.[73] The first bacterial genome sequenced in its entirety was that of *Haemophilus influenzae*, marking the beginning of a new approach to vaccine antigen discovery.[74] Since this initial bacterial genome sequence determination, genomic sequencing of pathogens has advanced exponentially. Over 300 bacterial genomes have now been sequenced, and hundreds more are currently in process. Genome-based antigen discovery is being applied to a wide range of bacteria, including streptococci, pneumococci, staphylococci and *Chlamydia*, as well as nonbacterial pathogens such as *Plasmodium falciparum*.[75]

An alternate, promising approach to novel antigen discovery has been built on technological advances in proteomics.[76, 77] These advances include development of high-resolution two-dimensional gel electrophoresis techniques and mass spectrometry methods that enable separation, identification, and purification of individual proteins from the complex mixture of proteins expressed by a pathogen. In proteomic analyses, a small culture of bacteria, preferably taken directly from an infected person, or otherwise grown in physiologically similar conditions, is subjected to physical or enzymatic treatment with specific proteases to generate peptide fragments that are then fractionated by a micro-high-performance liquid chromatography method and sequenced by molecular mass-by-mass spectrometry. An overlapping set of peptides of approximately 8–10 amino acids is sufficient to identify an antigen and provides the means to find the gene. Although proteomic analysis is, in some ways, more involved than genomic analysis, it provides an important new approach to antigen identification, and offers a direct way to document that the specific protein identified is actually expressed by the pathogen (including, for example, demonstration that a protein of

interest is expressed on the external surface of the pathogen).[78, 79] A combination of proteomic and serologic methods to select potential novel vaccine immunogens, called serological proteome analysis, or SERPA,[80, 81] can be used to screen the pathogen proteome for expressed proteins that are recognized by antibodies present in sera obtained from individuals who have recovered from an infection with the pathogen.

The proteomic approach to antigen discovery has been applied to identify novel vaccine candidates for a number of human pathogens, including *Helicobacter pylori*, *Chlamydia pneumoniae*, *Staphylococcus aureus*, *Bacillus anthracis*, *Haemophilus influenzae,* and *Plasmodium falciparum*. As in new genomic methods for antigen discovery, having identified a gene encoding a candidate antigen by proteomic methods, it is then necessary to show that an immune response of the desired type can be raised against the protein. In addition, it is necessary to show that immune responses elicited following immunization with the candidate antigen engender some degree of protection. Often, pure protein antigens produced by recombinant methods are not very immunogenic. As such, many of the emerging recombinant vaccines so produced will likely require enhancement of their immunogenicity by means of an adjuvant.

ADJUVANTS

The term 'adjuvant' (derived from the Latin *adjuvare*, to help) refers to any substance added as a component of a vaccine preparation – in addition to the vaccine antigens themselves – that improves the immunological response to the antigen. As such, 'adjuvant' is a catch-all term including a broad range of molecular entities that act via diverse – and, in a number of instances, yet to be elucidated – pathways. Until recently, most adjuvants were derived empirically and the mechanisms by which they augmented immune responses were unknown . As a result, there were few, if any, principles available to guide the improvement of known adjuvants or the development of new ones. However, recent advances in understanding of the mechanisms by which dendritic cells sense the presence of pathogens and their constituents, and translate this information to shape the quantity, quality, and durability of host cellular and humoral adaptive immune responses, have transformed adjuvant discovery and optimization. What was once a process of trial and error now represents an area of hypothesis-driven research and mechanism-based discovery.

A particularly promising advance emerged from the discovery that pathogen sensing by the innate immune system is mediated by recognition of specific pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) that are expressed on dendritic cells (DCs) and other hematolymphoid and some epithelial cells[82] (Chapter 3). The pathogen-derived PAMPS recognized by TLRs consist of structures that are found only in or on pathogens (including bacteria, viruses, and parasites) and are not part of normal vertebrate biology. Following binding of a specific PAMP to a specific PRR, a specific cellular activation and response cascade is triggered that can directly confront an intruding pathogen and/or lead to the activation of specific host adaptive immune response mechanisms. These breakthroughs in basic immunology have been readily translated into what can now be considered the science of adjuvant biology.[83, 84]

Such progress has occurred at an especially opportune time as new vaccine development strategies have transitioned from traditional approaches using attenuated or killed pathogens to highly defined and purified recombinant proteins (so-called "subunit" vaccines) or nonreplicating vectored antigens. Although these newer approaches are promising from the perspective of vaccine safety and the opportunity they afford to design the structures of vaccine immunogens, recombinant or synthetic vaccines are often inherently less immunogenic than traditional vaccines based on attenuated live viruses or intact killed organisms. In the context of current vaccine development and regulatory approval processes, an adjuvant is developed as part of a vaccine, not as an independent product. Consequently, there are currently no adjuvants licensed by regulatory authorities as stand-alone products.

Most contemporary efforts to develop novel adjuvants are focused on the targeted activation of TLRs that are expressed on specific cells critical for the generation of innate and adaptive host responses to specific pathogens.[85] A family consisting of 10 distinct TLRs has been identified to date in humans (Chapter 3). TLRs are expressed in a number of innate immune cells, including DCs, macrophages, neutrophils, endothelial cells, and fibroblasts. Given the importance of DCs as critical antigen-presenting cells, most studies of the biology of TLR signaling have focused on these cells. Different TLRs are expressed on distinct subpopulations of DCs, and, depending on the TLR, in distinct cellular compartments. TLRs expressed on the surface of human myeloid DCs include TLR2 (which is heterodimerized with TLR 1 or 6), as well as TLRs 4, 5, 6, and 10 (Fig. 92.6), while these same cells express TLRs 3 and 8 within endoplasmic reticulum (ER) and phagolysosomes. Plasmacytoid DCs (pDCs) express TLR7 and 9 within ER/phagolysosomes. The TLRs expressed on the cell surface are primarily activated by PAMPs encountered in the extracellular environment, while TLRs expressed in the ER/phagolysosomes are activated by PAMPs (including viral pathogen-derived RNA or DNA) that tend to be routed through these endosomal compartments. Activation of TLRs on innate immune cells leads to their production of specific cytokines, as well as their expression of co-stimulatory molecules, leading to induction of adaptive immune responses. Given that different DCs express different TLRs, and that signaling via different TLRs results in the expression of a distinct pattern of cytokines, it is believed that activation of specific TLRs can variously favor the induction of Th1- or Th2-biased immune responses, or can differentially augment either direct or cross-presentation pathways for antigen presentation (Fig. 92.6). Although most data on induction of specific types of immune responses by engagement of specific TLRs have emerged from murine studies (and have not yet been validated in humans), the ability to tailor an adjuvant preparation to achieve a desired type of immune response with a specific vaccine immunogen is a promising notion. Naturally occurring ligands for TLRs include lipopolysaccharide (LPS) from bacterial cell walls (recognized by TLR4), triacyl lipopeptides (recognized by TLRs 1 + 2), diacyl lipopeptides (recognized by TLRs 1 + 6), peptidoglycan (recognized by TLR2), flagellin (the monomer that makes up flagella, recognized by TLR5), single-stranded RNA (recognized by TLR7), double-stranded RNA (recognized by TLR3), and unmethylated DNA containing the dinucleotide pair CpG[86] (recognized by TLR9). Based on these insights, a variety of approaches to develop adjuvants predicated to activation of specific TLR pathways are being actively pursued.

One interesting aspect of adjuvant development is how it is revealing the mechanisms of action of adjuvants that were originally identified via a process of trial and error, as well as delineating important aspects by which certain empirically derived vaccines are able to induce high-level, long-lasting immune responses. One illustrative example can be found in the case of complete Freund's adjuvant (CFA), which has long served as

the benchmark for laboratory studies of adjuvants. CFA is a mixed emulsion of mineral oil, mannide monooleate, and killed mycobacteria. However, it is far too reactogenic for use in humans, causing significant pain and abcesses at the site of injection – reactions that would be exacerbated if CFA were to be used repeatedly. An alternative preparation termed incomplete Freund's adjuvant (IFA) lacks the mycobacterial component, but it too is associated with injection site reactions that are severe enough to limit its use to experimental therapeutic cancer vaccines. Although CFA's toxicity precludes its use as a vaccine adjuvant in humans, many of its constituents (including liposaccharides, DNA, and specific bacterial cell wall components) are now understood to exert their adjuvant effects on vaccine-induced immune responses via engagement of specific TLRs. Similarly, the live attenuated *Mycobacterium bovis* strain, BCG, long widely employed as a vaccine for the prevention of tuberculosis, includes cell wall, peptidoglycan, and DNA components that activate specific TLRs. Interestingly, the highly effective yellow fever vaccine 17D has been shown to activate multiple TLRs as part of its induction of antiviral immune responses.[87] It is quite likely that other live attenuated viruses that transiently replicate in immunized hosts also activate innate immune responses via engagement of TLRs. In yet another example involving a nonreplicating vaccine immunogen, one version of the Hib polysaccharide conjugate vaccines now licensed for use in children for the prevention of invasive Hib disease includes the meningococcal outer-membrane protein complex (OMPC) as its protein carrier. OMPC conjugates have favorable immunogenic properties that correlate with the ability of OMPC to activate DCs via TLR2.[88]

Hundreds of different adjuvant formulations have been tested in animal models, and a few have been advanced into human studies. With a few

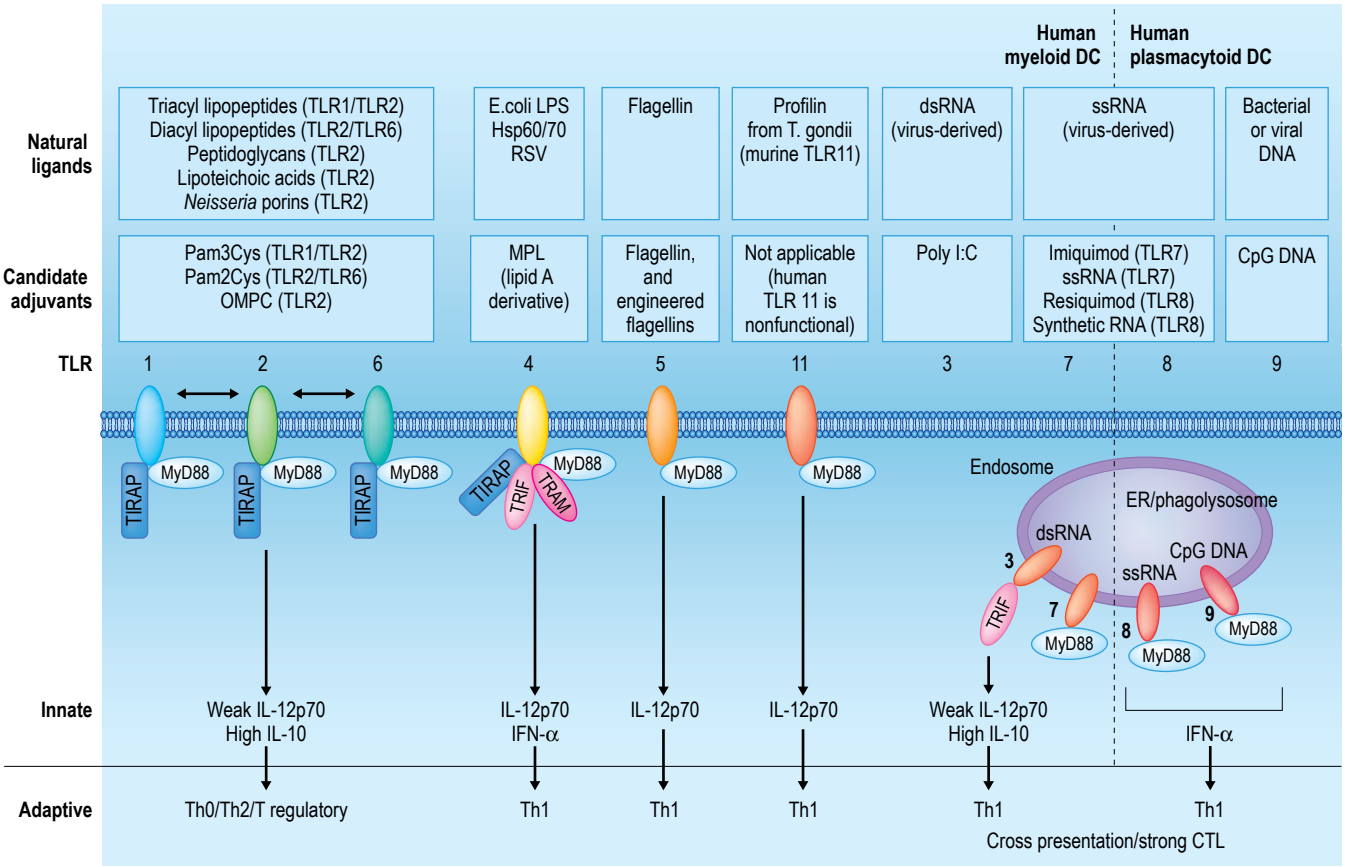

Fig. 92.6 Toll-like receptor (TLR) signaling pathways and mechanism-based adjuvants. The targeted activation of specific dendritic cell (DC) populations via engagement of specific TLRs to initiate innate and adaptive immune responses represents a very promising approach for the development of novel adjuvants based on natural or synthetic versions of the pathogen-associated molecular patterns (PAMPs) that trigger specific TLRs. Specific TLRs and their natural activating ligands are shown above. Different TLRs are associated with different adaptor proteins that propagate intracellular signaling along distinct pathways which favor specific immune responses (e.g., Th1, Th2, cross-presentation or CTL priming). The character of responses from specific TLR engagement illustrated is based on animal and ***ex vivo*** studies. In humans, TLRs 7 and 9 are expressed in the endoplasmic reticulum (ER)/phagolysosomes of plasmacytoid DCs (pDCs) that represent the major sources of type I interferon production (e.g., IFN-α). Human myeloid DCs (mDCs) express TLR3 (in the ER/phagolysosomes); and TLR2 (heterodimerized with TLRs 1 or 6), and TLRs 4, 5, 8, and 11 on the cell surface. (Adapted from Pulendran B, Ahmed R. Translating innate immunity into immunologic memory: implications for vaccine development. Cell 2006; 124: 849–863, with permission from Elsevier.

notable exceptions, these formulations have not yet been licensed as a vaccine. Major classes of adjuvants now available and in development include: (1) alum; (2) liposomes; (3) immune-stimulating complexes (ISCOMs); (4) virosomes; (5) emulsions; (6) cytokines; and (7) Toll-receptor agonists.

Alum

Alum, the classical adjuvant most often used in vaccines in humans, includes a range of salts of aluminum precipitated under basic conditions, usually aluminum sulfate mixed with sodium or potassium hydroxide plus a variable amount of phosphate.[89] The relative proportions will determine the size, charge, and solubility of alum. The composition of alum used as an adjuvant varies in currently available vaccines and may influence vaccine immunogenicity. Alum is utilized as an adjuvant in many of the currently available vaccines composed of inactivated toxins or recombinant proteins (live attenuated vaccines do not include alum or other adjuvants).

Alum serves two main purposes as an adjuvant. First, it acts as an antigen depot. Vaccine antigens adsorb to alum and elute from it following injection into the host. Second, alum acts a mild irritant, causing the recruitment of leukocytes necessary for generation of an immune response to the site of injection. Adsorption of antigens on to alum routinely improves immunogenicity, particularly the antibody response. Alum does not typically enhance CD8 T-cell responses. Alum has been a component of many vaccines for decades and has an excellent safety record. As new adjuvants are developed, alum may remain as a component of combination adjuvant mixtures (as is the case with some newer adjuvants now approaching clinical use), or it may eventually be supplanted by other agents that more effectively provide favorable depot and local inflammatory responses to accentuate host immune responses.

Liposomes

Using lipids with polar head groups (e.g., triglycerides) and differing types of hydrophobic tails, one can form either micelles (spheres) or multilamellar sheets in aqueous environments.[90] Under the right conditions, antigens can be incorporated into the spheres or between layers of the sheets, providing a potential slow-release depot system. Immunopotentiators such as QS21 or detoxified LPS derivatives (such as monophosphoryl lipid A (MPL)) may be added to the lipid mix.[45]

Immune-stimulating complexes

ISCOMs are a proprietary form of liposomes made of cholesterol, saponins from Quillaia bark (various members of the QS-X family of triterpene glycosides), and phospholipids that form cage-like structures into which antigens can be entrapped or intercalated.[86] ISCOM complexes may provide a depot function, as well as facilitate the delivery, uptake, and processing of vaccine immunogens by APCs.

Virosomes

Purified influenza virus hemagglutinin (HA) and neuraminidase mixed with phosphatidyl choline and phosphatidyl ethanolamine (polar lipids) will form empty particles that have the surface properties of influenza virus. Adding an antigen in solution before mixing the lipids results in the incorporation of the antigen inside the particle. This provides a vehicle for delivering antigens to the interior of a cell, via the influenza HA membrane fusion process, thereby enabling antigen processing and presentation via both major histocompatibility complex (MHC) class 1 and 2 pathways.[91]

Emulsions

Numerous oil-in-water and water-in-oil emulsions have been tested as adjuvants. One such emulsion, MF59, is used in a licensed influenza vaccine. MF59 consists of squalane, a metabolizable shark oil and two surfactants, polyoxyethylene sorbitan monooleate and sorbitan trioleate, in an oil-in-water emulsion.[92]

Cytokines

Cytokines are host-produced immunomodulators that regulate immune cell action (Chapter 10). Several cytokines are being tested as potential vaccine adjuvants, including granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), and IL-12.

Toll-receptor agonists

Of the defined TLR agonists being explored as vaccine adjuvants, LPS and its partially detoxified form, MPL, which activate TLR 4, have been most thoroughly explored in clinical trials. With evidence of enhanced ability to increase the percentage of individuals responding with protective antibody levels to hepatitis B as compared to a standard hepatitis B vaccine, one hepatitis B vaccine that employs an adjuvant formulation (termed AS04) consisting of a combination of alum and MPL[93] has been licensed for use in high-risk individuals. In addition, a vaccine against HPV that uses the same adjuvant formulation may be licensed soon, and candidate HSV-1 and malaria vaccines currently being studied in late-stage clinical trials also include this alum–MPL combination adjuvant.

A wide variety of TLR9-specific agonists consisting of oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) are being evaluated in preclinical studies. These CpG-ODNs resemble bacterial DNA, modified to include a phosphorothioate backbone to increase their stability. Two CpG-ODN adjuvants have been evaluated in recent phase I and II trials and shown to increase the timing and magnitude of induction of protective antibody levels, as well as the proportion of responding individuals, to recombinant HBSAg vaccine as compared with the current commercially available version of the vaccine.[94] One of these CpG-ODN adjuvants also elicits protective antibody responses in immunized HIV-infected individuals who had previously failed to respond to the hepatitis B vaccine. This approach is now being studied as a way of inducing protective immune responses to hepatitis B earlier after initiation of the vaccination regimen or with fewer doses of the vaccine.

In addition to the CpG-ODN-based TLR9 adjuvants described above, small chemical compounds with structures that resemble nucleic acid bases have been identified that activate TLR7 (e.g., imiquimod) or both TLR7 and 8 (e.g., resiquimod). These compounds are being evaluated as vaccine adjuvants in preclinical studies. Flagellin, a TLR5 agonist, is also being explored as an adjuvant.

Recently, attention has also been focused on coupling, rather than mixing, TLR agonists to antigens. CpG oligonucleotides conjugated to antigens have been tested in preclinical studies of hepatitis B vaccines[95] and in human clinical trials for treatment of allergy.[96] Ligands for TLR7/8 have been coupled to HIV antigens,[97] and the ligand for TLR5 (flagellin) has been fused to a variety of antigens.[98, 99] In some instances,

coupling a TLR ligand to an antigen resulted in a substantial improvement of the immune response compared to mixtures – potentially the result of enabling the antigen and the TLR ligand to co-locate in the same DC compartments.

Numerous preclinical studies have confirmed that many natural and synthetic TLR agonists possess adjuvant activity. Importantly, early human clinical trials of TLR-predicated adjuvants have supported the promise of this approach to mechanism-based strategies to augment vaccine immunogenicity. An important challenge is to define the most potent and best-tolerated variants, and to define rules by which activation of specific TLR pathways might translate into predictable augmentation of desired types of immune responses. It is hoped that general rules will emerge to suggest which of an increasing number of novel adjuvants in development performs best with which type of vaccine immunogen, and if results obtained with a specific type of immunogen–adjuvant combination can be extrapolated to predict the likelihood of enhanced immunogenicity with other vaccines. Although beyond the reach of available experimental results, the ability to tailor, titrate, and otherwise optimize immune responses to vaccines by manipulation of specific TLR pathways appears a realistic future possibility. However, important challenges remain. In particular, a primary challenge for next-generation adjuvant development is finding a combination that retains immunopotentiating action while minimizing vaccine-associated adverse experiences. Short-term adverse experiences, such as local injection site reactions, represent undesirable side effects that may disqualify candidate adjuvants early in clinical development. However, given that vaccines are administered to healthy people to prevent potential future infectious diseases, the potential for rarer adverse experiences (such as autoimmunity) that may only be manifest with much longer latency from the time of vaccine adjuvant administration will undoubtedly be important considerations for use in prophylactic vaccines.

NOVEL VACCINE VECTORS

As induction of cell-mediated immune responses is considered an important component of vaccine strategies for many diseases for which no vaccines are currently available (many of which are caused by intracellular pathogens), there is a need to develop safe and readily scalable approaches to elicit durable CD8 T-cell responses in immunized humans. Further, given the critical role that CD4 T cells play in induction, differentiation, and maintenance of CD8 T-cell responses, any such novel vaccine strategy will likely also require appropriate CD4 T-cell responses. As elicitation of CD8 T-cell responses against a foreign antigen usually depends on the *de novo* expression of the antigen within a host cell and its subsequent processing and presentation via class I MHC pathways (Chapter 6), most novel vaccine strategies are predicated on the need to achieve synthesis of pathogen-derived antigens within APCs of immunized human hosts. With an increasing appreciation of the role that cross-presentation pathways can play in elicitation of class I-restricted CD8 T-cell responses, such *de novo* antigen synthesis may not need to occur within APCs themselves (which may be an advantage for potential vaccine delivery strategies that do not directly target APCs).

One of the many attractive attributes of effective live attenuated vaccines is their ability to recapitulate (to various degrees) many of the processes that lead to the generation of potent immune responses following natural infection. These processes include the fact that replication of all viruses depends on gaining access to host cells for genome replication and for the synthesis of essential components of virus particles that permit further propagation of the infection within and between hosts. One immunologic benefit of this requirement is that *de novo* synthesis of viral gene products within infected cells provides a key opportunity for viral antigen presentation (via MHC class I pathways) and elicitation of antiviral cellular immune responses. Along with the processing and presentation of intact virus proteins via MHC class II pathways leading to production of antiviral antibody responses, live attenuated viral vaccines have a strong track record for induction of broad cellular and humoral immune responses that likely both contribute to conferring protective immunity. However, despite their track record of success, it is likely that few, if any, new live attenuated viral vaccines will be derived in a manner that resembles previous successful efforts (e.g., the empiric derivation of live attenuated polio, yellow fever, or varicella-zoster vaccines). Important reasons for this change include the desire for safe and well-characterized vaccines whose mechanisms of attenuation are defined and that can be monitored in the course of vaccine production and use. Indeed, most of the recently developed live attenuated vaccines were derived using new approaches for genetic reassortment (Fig. 92.4) wherein genome segments encoding pathogen-derived antigens of interest are recombined with a common set of genome segments that carry attenuating mutations (derived either by use of attenuating viral passage under specific conditions in cell culture (e.g., the cold-adapted influenza vaccine or use of a virus obtained from a nonhuman host that is itself inherently unable to replicate to high levels in humans, e.g., the reassortant rotavirus vaccine prepared via genetic reassortment between human and bovine rotavirus strains (Fig. 92.4)[100]). Although such approaches have proven successful, they are limited in that they can only be applied to homologous viruses (e.g., those derived from the same virus type) whose genomes are segmented and capable of ready genetic reassortment in culture, or to viruses that can be manipulated by reverse genetics.

In response to the desire to produce vaccines that can safely and reliably elicit desired immune responses, especially T-cell responses, several approaches are being explored to develop novel vector systems that permit the expression of pathogen-derived antigens. As many of these approaches are based on viruses distinct from the viral pathogen targeted for induction of host immune responses, the inserted pathogen-derived gene products are expressed via recombinant methods as heterologous antigens. Alternatively, in nonviral expression systems, such as DNA vaccines, the pathogen-derived antigen is expressed in isolation and does not depend on virus-mediated antigen delivery to APCs following host inoculation.

Collectively, such recombinant heterologous expression systems are commonly referred to as 'vaccine vectors.' In some instances, such recombinant vectors express only a specific antigen (in the case of DNA vaccines or certain viral vectors, e.g., adenovirus), while in others both the inserted pathogen-derived antigens and antigens encoded by the viral vector 'backbone' are expressed (e.g., poxvirus vectors). Most new approaches employ expression systems that are inherently nonreplicating (e.g., DNA vaccines) or that employ viral vectors that can replicate at high levels in tissue culture but not *in vivo* (e.g., complemented adenovirus deletion variants or host range-restricted poxviruses). While numerous approaches are being pursued to develop novel vaccine vectors, they will all need to meet certain common criteria to emerge as vaccine approaches applicable for widespread use. In particular, any successful approach must be safe in healthy and immunodeficient humans (given

their increased representation in the population as a result of HIV infection and therapeutic immunosuppression), desirably immunogenic (including in individuals who may have been previously exposed to the virus from which the vector was derived, e.g., vaccinia or adenovirus), and able to be produced in large quantities and in a stable manner. A limited number of vaccine vector approaches now being pursued are likely to meet these criteria.

Should successful approaches emerge, there will likely be interest in applying them for use in vaccines targeting diverse pathogens. Thus, while definition of promising, broadly applicable, vaccine vector approaches may help simplify certain aspects of vaccine regulatory review and manufacture, they may also present challenges to prioritize use for specific applications should administration of a given vaccine vector on one occasion compromise or preclude successful administration at a later time. Nevertheless, the development of novel vaccine vectors is laying essential groundwork for the development of next-generation vaccines.

Several novel vaccine vectors currently being studied in preclinical studies and human clinical trials are described below, all of which depend on the delivery and expression of a candidate pathogen-derived gene sequence. In a number of ways, DNA vaccines represent the simplest approach to deliver pathogen-derived genes. Viral vectors similarly serve to deliver pathogen gene sequences to host APCs, either directly or indirectly, but do so in a manner that depends on and takes advantage of the lifecycle and tropism of the virus that is being adapted to express the exogenous pathogen gene products.

DNA vaccines

The ability of purified plasmid DNA containing heterologous antigens expressed under the control of eukaryotic transcriptional regulatory and RNA processing signals to elicit immune responses when injected into experimental animals was discovered serendipitously.[101] However, since the initial, quite surprising, description, the development of so-called DNA vaccines has become an active area of preclinical and clinical vaccine development.[102] Reasons for this enthusiasm include the attractive simplicity and facile preparation of vectors that encode only the defined antigen of interest (which can itself be manipulated via recombinant methods to assume a desired configuration), a reasonably straightforward method for vaccine production, and the inherent stability to temperature (which is much greater than most currently live or subunit vaccines). Although the DNA vector is most commonly injected intramuscularly, the generation of specific immune responses depends on the uptake of the vector DNA by APCs followed by the expression, processing, and presentation of vector-encoded antigens. As tissue and tissue fluids present a hostile environment for purified DNA, and the process of DNA uptake by APCs appears to be relatively inefficient, much of the dose of injected DNA is degraded before it can be reached by an APC that can initiate the desired immune response.

Most DNA vaccine research has been pursued in mice, although studies have now been performed in numerous animal species. Studies have usually utilized intramuscular injection of vaccine vector DNA, but various intradermal and transdermal approaches have also been explored. Murine studies have shown that administration of antigen-encoding plasmid DNA can elicit appreciable cellular and humoral immune responses that may confer protection against experimental challenge. However, translation of these promising results in animal models to humans has proven frustrating. While DNA vaccines have been generally well tolerated in immunized volunteers, in most human studies of DNA vaccines, administration of even substantial quantities of DNA vaccine vectors has elicited relatively low-level immune responses. It is not yet known whether these disappointing results reflects fundamental differences in the immunogenic behavior of DNA vaccines in humans and mice, or the fact that the DNA doses administered to humans do not match those administered to mice (DNA per weight of the immunized host). Given the substantial size differences between humans and mice, it would likely be impractical (for reasons of both vaccine supply and the actual process of administration of sufficiently high doses of DNA) to administer the relative murine dose to humans. As such, a variety of approaches are being explored to prolong DNA survival in tissue, promote more efficient targeting of DNA to APCs, or to develop novel adjuvants that might specifically amplify immune responses to DNA vaccines.[103, 104]

DNA vaccines are currently being used as candidate preventive vaccines for a wide variety of infectious diseases, including HIV, tuberculosis, malaria, and CMV.

Poxviruses

Poxviruses represent the family of viruses that are physically the largest viruses and that possess the largest genomes. Much of the poxvirus genome encodes gene products that serve to evade host immune responses, and that are not required for virus replication in tissue culture. Further, facile techniques for the insertion and deletion of specific viral genes have been developed. The ability to accommodate sizeable foreign gene inserts is, in part, a function of the large size of the poxvirus genome (and the large packaging capacity of poxvirus virions). As a result of these favorable attributes, poxviruses have been utilized extensively in laboratory studies of virus biology, recombinant protein production, and host immune responses.[105] Although poxviruses encode multiple gene products that help the virus evade host immune responses, they are, nevertheless, potent immunogens. Studies of individuals immunized decades ago with vaccinia virus (in the course of smallpox eradication efforts) have shown that this virus induces long-lasting memory T- and B-cell immune responses.

In contrast to most of the other viral vectors currently being developed, poxviruses can replicate readily in culture and do not require an engineered host cell to support propagation *ex vivo*. One important limitation of all poxvirus vectors developed to date is that, given the large size of the poxvirus genomes and the multitude of gene products they naturally express, even large inserts derived from foreign pathogens of interest will present only a minority of the vaccine vector antigens delivered to and recognized by the host immune system. To be effective, approaches to focus immune responses on the antigen of interest will need to be developed. Toward this end, a variety of so-called 'prime–boost' [106] approaches are being explored where the host immune response is primed with one type of recombinant vaccine vector (such as a DNA vaccine or adenovirus vector) and then boosted with subsequent delivery of poxvirus vectors encoding the same antigen. In this manner, immune responses to antigens of interest have been significantly augmented in a number of preclinical studies.

Vaccinia virus represents the prototypic vaccine vector. This virus is the same one that was employed in the successful smallpox eradication campaign, and has been used as a laboratory tool for decades. However, given current high expectations for vaccine safety, and the increased number of immunodeficient individuals present in the population (as a result of the

emergence of the HIV pandemic and the increased use of immunosuppressive therapies in clinical medicine) at high risk of serious adverse events, and potentially fatal consequences, from vaccinia immunization, the original vaccinia strains used in smallpox eradication efforts are not considered safe for general use. However, studies of vaccinia-based vaccine vectors have provided a strong basic foundation for research on other more highly attenuated poxvirus variants.

Modified vaccinia Ankara (MVA) is an attenuated vaccinia virus that was originally derived by prolonged passage of a vaccinia virus isolate on chicken embryo fibroblasts in culture. In the course of extensive passage in culture, a viral variant emerged that had fortuitously deleted large sections of the viral genome, including those that encode important poxvirus immune evasion genes and those that determine the ability of the virus to replicate on cells obtained from different animal species. Specifically, while MVA grows well on chicken cells, it cannot replicate in human cells in culture or *in vivo*, conferring an inherent safety feature.

MVA was safely administered to over 100 000 individuals at high risk of adverse consequence for vaccinia immunization toward the end of the smallpox eradication effort. More recently, it has garnered renewed interest as a potential safer smallpox vaccine in the wake of concerns about bioterrorism threats. Even though MVA cannot replicate in mammalian cells, the virus demonstrates favorable immunogenic properties. MVA has been used as a vector expressing genes for a wide variety of genes, including HIV and malaria antigens either alone or, as described above, in 'prime–boost' regimens, where MVA has been administered following initial priming immunizations with other vaccine vectors. A concerted effort is under way to improve further the performance of MVA by manipulating a series of poxvirus genes that dampen the human immune response to the virus (and to any antigens inserted in it).[107]

Avipox is a family of poxviruses that infect birds and cause respiratory diseases in poultry. Canarypox, a member of the avipox group, has been adapted as a vaccine vector. Canarypox replicates well on avian cells in culture but cannot replicate on human cells in culture or in humans *in vivo*. As a result, canarypox, like MVA, provides an interesting vector system with inherent safety features.[108] Canarypox vectors carrying HIV genes have been tested in several clinical studies, either alone, or in 'prime–boost' regimens following priming with adenovirus vectors and recombinant protein antigens. In a large ongoing phase III HIV vaccine trial, a recombinant canarypox vector is being used as a priming vector, followed by boosting with a recombinant version of the HIV gp120 surface Env protein. To date, the results from human clinical trials of canarypox vectors have been disappointing, with only low-level specific immune responses generated in human volunteers.[109]

Adenoviruses

Adenoviruses, one of the common causes of upper respiratory and gastrointestinal infections, have seen extensive use in clinical trials and were one of the first gene therapy vectors.[110] Most adenovirus vectors currently being studied in preclinical and clinical settings are disabled by deletion of the early E1 genes that are necessary for replication in an immunized host. Most adenovirus vaccine vectors developed have used the well-characterized and readily produced adenovirus serotype 5 (Ad5) as the vector 'backbone.' Disabled adenovirus vectors are grown in cells that express the E1 genes artificially inserted into the cell's genome.[111] Once these disabled vectors, encoding a heterologous pathogen-derived antigen of interest, enter a cell, the pathogen gene product is expressed, processed, and presented by host APCs. As adenoviruses can directly infect dendritic cells, they promise to provide efficient vaccine vectors. Robust antibody and CD8 T-cell responses to heterologous antigen genes expressed by adenovirus vectors have been observed in preclinical animal models. Furthermore, in early-phase human clinical trials, adenovirus vectors have been generally well tolerated, and proven to be the most effective of any recombinant vector system studied to date in eliciting high-level CD8 T-cell responses.

The main potential drawback to widespread use of adenovirus vectors in humans is that, depending on the adenovirus type and the geographic location, variable levels of pre-existing immunity are found in humans as a result of prior naturally acquired adenovirus infections. High levels of antibody against the adenovirus vector might blunt the immunogenicity and efficacy of an adenovirus vector-based vaccine, but it remains to be seen if this will be a significant limitation.[112] Should pre-existing immunity to adenovirus vectors derived from epidemiologically prevalent serotypes (e.g., Ad5) limit vaccine immunogenicity, current efforts to develop vaccine vectors based on serotypes that are rare in human populations or novel adenovirus vectors specifically designed to avoid pre-existing antibody responses may yield effective alternative approaches.

Adenovirus vectors are currently used in clinical trials for vaccines against HIV,[113] malaria, influenza, and a range of other pathogens.

Alphaviruses

Alphaviruses are RNA viruses that cause zoonotic diseases, such as Venezuelan equine encephalitis. These viruses do not normally circulate in humans, so immunity to these viruses is quite rare in humans. Alphaviruses have a strategy for overexpressing the proteins that make up the virion by making a separate subgenomic RNA specifically encoding these gene products. Current recombinant alphavirus vaccine vector strategies take advantage of this subgenomic transcript, replacing the viral genes with selected genes for other antigens, but maintaining the signals for translation and protein production. In addition, through use of genetic complementation, it is possible to generate virus particles that only contain this heterologous antigen-encoding expression cassette. Such virus particles can efficiently mediate infection of host cells, but because they lack other alphavirus genes needed for virus replication cannot spread beyond the initial target cell infected.[114, 115] Alphavirus vectors rival the adenoviruses in efficiency of protein production in tissue culture and have induced robust antibody and T-cell responses in preclinical studies.[116] One current limitation of the alphavirus vector system is the difficulty of scaling the production system; however, this is a technical matter that should be addressable. In addition, ample safety data will be needed before widespread use of alphavirus vaccines achieves endorsement by regulatory authorities for use in healthy populations.

Adeno-associated virus

Adeno-associated viruses (AAV) belong to a family of single-stranded DNA viruses (parvoviruses) that include the B19 parvovirus that causes a rash in children known as 'fifth disease' (measles, mumps, rubella, and varicella make up the first four). AAV is transmitted in conjunction with adenovirus infection, and is not known to cause any significant disease. It is poorly immunogenic in the course of natural infections.[117] AAV can integrate into the genome of the infected cell, usually in a particular place on chromosome 19, although integration

KEY CONCEPTS

VACCINE APPROACHES BEING EXPLORED TO IMPROVE ON NATURAL IMMUNITY

For many important infectious diseases for which no vaccines are currently available, successful derivation of effective vaccines will depend on improving upon natural immunity, especially in those instances where natural immunity does not follow natural infection (such as human immunodeficiency virus (HIV) and malaria) or where safety concerns limit the development of specific protective antigens (*Neisseria meningitidis* group B). In addition, for other pathogens that typically manifest significant genetic (and antigenic) diversity (such as influenza, HIV, and bacteria such as *Streptococcus pneumoniae*), a need exists to develop novel vaccines that can protect against a wide range of variants with a limited number of vaccine immunogens. Towards these ends, a number of new approaches, enabled by new vaccine technologies, are being pursued, including:

- Targeted alteration of protective antigens to increase their ability to elicit protective immune responses (e.g., efforts to alter the structure of the HIV Env glycoproteins gp120 and gp41 so that they elicit higher-level, more potent, neutralizing antibody responses than their native counterparts)
- The development of synthetic consensus antigens able to elicit broader immune responses than would sequences obtained from individual pathogen isolates (e.g., efforts to develop consensus immunogens able to elicit cytotoxic T-lymphocyte (CTL) responses against genetically diverse HIV-1 variants)
- Techniques for new antigen discovery to identify novel conserved antigens within otherwise genetically diverse pathogens (e.g., *Streptococcus pneumoniae*) or those for which currently known protective antigens cannot be developed as vaccines (e.g., *Neisseria meningitidis* group B)
- The use of novel adjuvants or vaccine vectors to enable generation of higher-level and/or more functional immune responses to pathogen antigens by vaccination than are seen following natural infection, and to enable high-level, fully functional memory immune responses to be activated at the time of initial infection (e.g., efforts to elicit high-level HIV-specific or hepatitis C virus-specific CTLs by recombinant viral vectors)
- Use of novel methods to shift relative immunodominance of specific pathogen gene products to increase the immunogenicity of conserved antigens from otherwise diverse pathogen genomes that are typically poorly immunogenic in the course of natural infections (e.g., efforts to augment the antibody response to the influenza A virus M2 protein via the use of potent adjuvants or conjugation to immunogenic carrier proteins)

does not appear to be efficient or site-specific when replication-defective adenoviruses of the type being developed as vaccine vectors are used. The propensity for chromosomal integration and poor immune response to the virus made AAV a good candidate for gene therapy; cells with an integrated viral genome could deliver a gene product for a long time without the immune system killing the infected cell. Recently, efforts have been made to adapt replication-defective AAV as a vaccine vector. Although encouraging results have been reported in preclinical studies, phase I studies in humans have demonstrated disappointing immunogenicity.

SUMMARY

The challenges to optimizing the full public health potential of existing vaccines largely relate to programmatic considerations. In contrast, the terrible impact of infectious diseases that cannot now be prevented by vaccines (such as the 'big three' killers of HIV, tuberculosis, and malaria) pose direct challenges to the scientific community to develop new generations of vaccines that overcome the largely biological obstacles to control and elimination of these diseases. The nature of the challenges posed by such pathogens necessitates that future vaccine efforts will not simply recapitulate the immune responses engendered by natural infection (as has been the premise of traditional vaccine development efforts), but rather, substantially improve upon them.

As the development of vaccines to prevent infections with the so-far refractory pathogens is pursued, improved understanding of the immune response to natural infection, as well as delineation of the reasons why host immune responses fail either to clear incipient infections or prevent future new ones, will be essential. Fortunately, early empiric approaches have now been replaced with hypothesis-driven strategies enabled by improved insight into the functioning of the human immune system, as well as new technologies, including higher-resolution tools to describe and quantitate pathogen-specific immune responses; novel methods for antigen discovery and targeted optimization of immunogenicity; the development of new, mechanism-based adjuvants; and the advent of innovative methods for vaccine vector-mediated antigen delivery. Thus, although the challenges may be vexing, the scientific and technical foundations on which vaccine development efforts rest have never been stronger.

REFERENCES

1. Ten great public health achievements – United States, 1900–1999. MMWR Morb Mortal Wkly Rep 1999; 48: 241–243.
2. Henderson DA. Principles and lessons from the smallpox eradication programme. Bull World Health Organ 1987; 65: 535–546.
3. Berndt ER, Glennerster R, Kremer MR, et al. Advance market commitments for vaccines against neglected diseases: estimating costs and effectiveness. Health Econ 2007; 16: 491–511.
4. Heymann DL, Aylward RB. Mass vaccination: when and why. Curr Top Microbiol Immunol 2006; 304: 1–16.
5. Anderson RM, May RM. Immunisation and herd immunity. Lancet 1990; 335: 641–645.
6. Peter G, Gardner P. Standards for immunization practice for vaccines in children and adults. Infect Dis Clin North Am 2001; 15: 9–19.
7. Zhou F, Santoli J, Messonnier ML, et al. Economic evaluation of the 7-vaccine routine childhood immunization schedule in the United States, 2001. Arch Pediatr Adolesc Med 2005; 159: 1136–1144.

8. Coffield AB, Maciosek MV, McGinnis JM, et al. Priorities among recommended clinical preventive services. Am J Prev Med 2001; 21: 1–9.
9. Cross AS, Opal S, Cook P, et al. Development of an anti-core lipopolysaccharide vaccine for the prevention and treatment of sepsis. Vaccine 2004; 22: 812–817.
10. Ruiz-Palacios GM, Perez-Schael I, Velazquez FR, et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. N Engl J Med 2006; 354: 11–22.
11. Vesikari T, Matson DO, Dennehy P, et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. N Engl J Med 2006; 354: 23–33.
12. Stewart AJ, Devlin PM. The history of the smallpox vaccine. J Infect 2006; 52: 329–334.
13. Tizard I. Grease, anthraxgate, and kennel cough: a revisionist history of early veterinary vaccines. Adv Vet Med 1999; 41: 7–24.
14. Dowdle WR, De Gourville E, Kew OM, et al. Polio eradication: the OPV paradox. Rev Med Virol 2003; 13: 277–291.
15. Falk LA, Chandler DK, Richman P. Review of current preclinical testing strategies for bacterial vaccines. Dev Biol Stand 1998; 95: 25–29.
16. Kreeftenberg JG. Standardization of acellular pertussis vaccines. Biologicals 1999; 27: 115–117.
17. Lesinski GB, Westerink MA. Vaccines against polysaccharide antigens. Curr Drug Targets Infect Disord 2001; 1: 325–334.
18. Lockhart S. Conjugate vaccines. Exp Rev Vaccines 2003; 2: 633–648.
19. Hilleman MR. Newer directions in vaccine development and utilization. J Infect Dis 1985; 151: 407–419.
20. Zuckerman AJ. The development of novel hepatitis B vaccines. Biochem Soc Symp 1987; 53: 39–49.
21. Kirnbauer R, Booy F, Cheng N, et al. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci USA 1992; 89: 12180–12184.
22. Garland SM, Hernandez-Avila M, Wheeler CM, et al. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. N Engl J Med 2007; 356: 1928–1943.
23. Villa LL, Ault KA, Giuliano AR, et al. Immunologic responses following administration of a vaccine targeting human papillomavirus types 6, 11, 16, and 18. Vaccine 2006; 24: 5571–5583.
24. Shaw AR. The rotavirus vaccine saga. Annu Rev Med 2006; 57: 167–180.
25. Parashar UD, Hummelman EG, Bresee JS, et al. Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis 2003; 9: 565–572.
26. Simonsen L, Morens D, Elixhauser A, et al. Effect of rotavirus vaccination programme on trends in admission of infants to hospital for intussusception. Lancet 2001; 358: 1224–1229.
27. Matson DO. RotaShield: the ill-fated rhesus-human reassortant rotavirus vaccine. Pediatr Ann 2006; 35: 44–50.
28. Geier DA, Geier MR. A review of the Vaccine Adverse Event Reporting System database. Exp Opin Pharmacother 2004; 5: 691–698.
29. Verstraeten T, DeStefano F, Chen RT, et al. Vaccine safety surveillance using large linked databases: opportunities, hazards and proposed guidelines. Exp Rev Vaccines 2003; 2: 21–29.
30. Griffith AH. Permanent brain damage and pertussis vaccination: is the end of the saga in sight? Vaccine 1989; 7: 199–210.
31. Afzal MA, Ozoemena LC, O'Hare A, et al. Absence of detectable measles virus genome sequence in blood of autistic children who have had their MMR vaccination during the routine childhood immunization schedule of UK. J Med Virol 2006; 78: 623–630.
32. Burgess DC, Burgess MA, Leask J. The MMR vaccination and autism controversy in United Kingdom 1998–2005: inevitable community outrage or a failure of risk communication? Vaccine 2006; 24: 3921–3928.
33. Tsai CM. Molecular mimicry of host structures by lipooligosaccharides of *Neisseria meningitidis*: characterization of sialylated and nonsialylated lacto-*N*-neotetraose (Galbeta1-4GlcNAcbeta1-3Galbeta1-4Glc) structures in lipooligosaccharides using monoclonal antibodies and specific lectins. Adv Exp Med Biol 2001; 491: 525–542.
34. Healy CM, Baker CJ. Prospects for prevention of childhood infections by maternal immunization. Curr Opin Infect Dis 2006; 19: 271–276.
35. Johnston MI, Fauci AS. An HIV vaccine – evolving concepts. N Engl J Med 2007; 356: 2073–2081.
36. Garber DA, Silvestri G, Feinberg MB. Prospects for an AIDS vaccine: three big questions, no easy answers. Lancet Infect Dis 2004; 4: 397–413.
37. Johnson VA, Brun-Vezinet F, Clotet B, et al. Update of the drug resistance mutations in HIV-1: 2004. Top HIV Med 2004; 12: 119–124.
38. HIV gp120 vaccine – VaxGen: AIDSVAX, AIDSVAX B/B, AIDSVAX B/E, HIV gp120 vaccine – Genentech, HIV gp120 vaccine AIDSVAX – VaxGen, HIV vaccine AIDSVAX – VaxGen. Drugs R D 2003; 4: 249–253.
39. Shiver JW, Emini EA. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. Annu Rev Med 2004; 55: 355–372.

39a. Steinbrook R. One step forward, two steps back — will there ever be an AIDS vaccine? N Engl J Med 2007; 357: 2653–2655.

40. Smith TA, Leuenberger R, Lengeler C. Child mortality and malaria transmission intensity in Africa. Trends Parasitol 2001; 17: 145–149.
41. Snow RW, Trape JF, Marsh K. The past, present and future of childhood malaria mortality in Africa. Trends Parasitol 2001; 17: 593–597.
42. Malaguarnera L, Musumeci S. The immune response to *Plasmodium falciparum* malaria. Lancet Infect Dis 2002; 2: 472–478.
43. Achtman AH, Bull PC, Stephens R, et al. Longevity of the immune response and memory to blood-stage malaria infection. Curr Top Microbiol Immunol 2005; 297: 71–102.
44. Ballou WR. Malaria vaccines in development. Exp Opin Emerg Drugs 2005; 10: 489–503.
45. Bojang KA. RTS,S/AS02A for malaria. Exp Rev Vaccines 2006; 5: 611–615.
46. Wizel B, Houghten RA, Parker KC, et al. Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2. J Exp Med 1995; 182: 1435–1445.
47. Maher D, Raviglione M. Global epidemiology of tuberculosis. Clin Chest Med 2005; 26: 167–182. v.
48. Skeiky YA, Sadoff JC. Advances in tuberculosis vaccine strategies. Nat Rev Microbiol 2006; 4: 469–476.

49. Fine PE, Rodrigues LC. Modern vaccines. Mycobacterial diseases. Lancet 1990; 335: 1016–1020.
50. Orme IM. Tuberculosis vaccines: current progress. Drugs 2005; 65: 2437–2444.
51. Orme IM. Preclinical testing of new vaccines for tuberculosis: a comprehensive review. Vaccine 2006; 24: 2–19.
52. de Jonge MI, Brosch R, Brodin P, et al. Tuberculosis: from genome to vaccine. Exp Rev Vaccines 2005; 4: 541–551.
53. Fulginiti VA, Eller JJ, Sieber OF, et al. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am J Epidemiol 1969; 89: 435–448.
54. Wilson S, Aprile MA. Sensitizing versus immunizing properties of inactivated measles vaccine. Prog Immunobiol Stand 1970; 4: 657–660.
55. Schmidt AC, McAuliffe JM, Murphy BR, et al. Recombinant bovine/human parainfluenza virus type 3 (B/HPIV3) expressing the respiratory syncytial virus (RSV) G and F proteins can be used to achieve simultaneous mucosal immunization against RSV and HPIV3. J Virol 2001; 75: 4594–4603.
56. Pichichero ME. The new meningococcal conjugate vaccine. A profile of its safety, efficacy, and indications for use. Postgrad Med 2006; 119: 47–54, 64.
57. Finne J, Leinonen M, Makela PH. Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. Lancet 1983; 2: 355–357.
58. Schrag S, Gorwitz R, Fultz-Butts K, et al. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR Recomm Rep 2002; 51: 1–22.
59. Mikkelsen M, Bukh J. Current status of a hepatitis C vaccine: encouraging results but significant challenges ahead. Curr Infect Dis Rep 2007; 9: 94–101.
60. Ferenczy MW. Prophylactic vaccine strategies and the potential of therapeutic vaccines against herpes simplex virus. Curr Pharm Des 2007; 13: 1975–1988.
61. Stanberry LR. Clinical trials of prophylactic and therapeutic herpes simplex virus vaccines. Herpes 2004; 11(Suppl. 3): 161A–169A.
62. Plotkin SA, Huygelen C. Cytomegalovirus vaccine prepared in WI-38. Dev Biol Stand 1976; 37: 301–305.
63. Adler SP, Plotkin SA, Gonczol E, et al. A canarypox vector expressing cytomegalovirus (CMV) glycoprotein B primes for antibody responses to a live attenuated CMV vaccine (Towne). J Infect Dis 1999; 180: 843–846.
64. Zhong J, Khanna R. Vaccine strategies against human cytomegalovirus infection. Exp Rev Anti Infect Ther 2007; 5: 449–459.
65. Moutschen M, Leonard P, Sokal EM, et al. Phase I/II studies to evaluate safety and immunogenicity of a recombinant gp350 Epstein–Barr virus vaccine in healthy adults. Vaccine 2007; 25: 4697–4705.
66. Stephenson JR. Understanding dengue pathogenesis: implications for vaccine design. Bull World Health Organ 2005; 83: 308–314.
67. Whitehead SS, Blaney JE, Durbin AP, et al. Prospects for a dengue virus vaccine. Nat Rev Microbiol 2007; 5: 518–528.
68. Ramos BV. A method for the screening of fusion protein expression by lambda-GT11 recombinant clones without the preparation of lysogens. Nucleic Acids Res 1989; 17: 6421.
69. Jones P. Antibody screening of bacteriophage lambda gt-11 DNA expression libraries. Methods Mol Biol 1998; 80: 439–447.
70. Wack A, Rappuoli R. Vaccinology at the beginning of the 21st century. Curr Opin Immunol 2005; 17: 411–418.
71. Rappuoli R. Reverse vaccinology, a genome-based approach to vaccine development. Vaccine 2001; 19: 2688–2691.
72. Muzzi A, Masignani V, Rappuoli R. The pan-genome: towards a knowledge-based discovery of novel targets for vaccines and antibacterials. Drug Discov Today 2007; 12: 429–439.
73. Grandi G. Genomics and proteomics in reverse vaccines. Methods Biochem Anal 2006; 49: 379–393.
74. Tang CM, Hood DW, Moxon ER. *Haemophilus* influence: the impact of whole genome sequencing on microbiology. Trends Genet 1997; 13: 399–404.
75. Gardner MJ, Hall N, Fung E, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 2002; 419: 498–511.
76. Nilsson CL. Bacterial proteomics and vaccine development. Am J Pharmacogenomics 2002; 2: 59–65.
77. Klade CS. Proteomics approaches towards antigen discovery and vaccine development. Curr Opin Mol Ther 2002; 4: 216–223.
78. Hillen N, Stevanovic S. Contribution of mass spectrometry-based proteomics to immunology. Exp Rev Proteomics 2006; 3: 653–664.
79. Strong M, Goulding CW. Structural proteomics and computational analysis of a deadly pathogen: combating *Mycobacterium tuberculosis* from multiple fronts. Methods Biochem Anal 2006; 49: 245–269.
80. Chitlaru T, Gat O, Grosfeld H, et al. Identification of in vivo-expressed immunogenic proteins by serological proteome analysis of the *Bacillus anthracis* secretome. Infect Immun 2007; 75: 2841–2852.
81. Klade CS, Voss T, Krystek E, et al. Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. Proteomics 2001; 1: 890–898.
82. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002; 20: 197–216.
83. Blander JM, Medzhitov R. On regulation of phagosome maturation and antigen presentation. Nat Immunol 2006; 7: 1029–1035.
84. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. Cell 2006; 124: 849–863.
85. Medzhitov R, Janeway CA Jr. Innate immune induction of the adaptive immune response. Cold Spring Harb Symp Quant Biol 1999; 64: 429–435.
86. Kersten GF, Crommelin DJ. Liposomes and ISCOMs. Vaccine 2003; 21: 915–920.
87. Querec TD, Pulendran B. Understanding the role of innate immunity in the mechanism of action of the live attenuated yellow fever vaccine 17D. Adv Exp Med Biol 2007; 590: 43–53.
88. Latz E, Franko J, Golenbock DT, et al. *Haemophilus influenzae* type b-outer membrane protein complex glycoconjugate vaccine induces cytokine production by engaging human Toll-like receptor 2 (TLR2) and requires the presence of TLR2 for optimal immunogenicity. J Immunol 2004; 172: 2431–2438.
89. Hem SL, White JL. Structure and properties of aluminum-containing adjuvants. Pharm Biotechnol 1995; 6: 249–276.

90. Desjardins R, Krzystyniak K, Therien HM, et al. Immunoactivating potential of multilamellar liposome vesicles (MLV) in murine popliteal lymph node (PLN) test. Int J Immunopharmacol 1995; 17: 367–374.

91. Gluck R, Burri KG, Metcalfe I. Adjuvant and antigen delivery properties of virosomes. Curr Drug Deliv 2005; 2: 395–400.

92. Atmar RL, Keitel WA, Patel SM, et al. Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. Clin Infect Dis 2006; 43: 1135–1142.

93. Kundi M. New hepatitis B vaccine formulated with an improved adjuvant system. Exp Rev Vaccines 2007; 6: 133–140.

94. Cooper CL, Davis HL, Angel JB, et al. CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. Aids 2005; 19: 1473–1479.

95. Payette PJ, Ma X, Weeratna RD, et al. Testing of CpG-optimized protein and DNA vaccines against the hepatitis B virus in chimpanzees for immunogenicity and protection from challenge. Intervirology 2006; 49: 144–151.

96. Broide DH. Immunostimulatory sequences of DNA and conjugates in the treatment of allergic rhinitis. Curr Allergy Asthma Rep 2005; 5: 182–185.

97. Wille-Reece U, Flynn BJ, Lore K, et al. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and $CD8^+$ T cell responses in nonhuman primates. Proc Natl Acad Sci USA 2005; 102: 15190–15194.

98. Huleatt JW, Jacobs AR, Tang J, et al. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine 2007; 25: 763–775.

99. McDonald WF, Huleatt JW, Foellmer HG, et al. A west nile virus recombinant protein vaccine that coactivates innate and adaptive immunity. J Infect Dis 2007; 195: 1607–1617.

100. Clark HF, Offit PA, Ellis RW, et al. The development of multivalent bovine rotavirus (strain WC3) reassortant vaccine for infants. J Infect Dis 1996; 174(Suppl 1): S73–S80.

101. Wells DJ. Intramuscular injection of plasmid DNA. Mol Cell Biol Hum Dis Ser 1995; 5: 83–103.

102. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259: 1745–1749.

103. Liu MA, Wahren B, Karlsson Hedestam GB. DNA vaccines: recent developments and future possibilities. Hum Gene Ther 2006; 17: 1051–1061.

104. Laddy DJ, Weiner DB. From plasmids to protection: a review of DNA vaccines against infectious diseases. Int Rev Immunol 2006; 25: 99–123.

105. Franchini G, Gurunathan S, Baglyos L, et al. Poxvirus-based vaccine candidates for HIV: two decades of experience with special emphasis on canarypox vectors. Exp Rev Vaccines 2004; 3(Suppl): S75–S88.

106. Kent S, De Rose R, Rollman E. Drug evaluation: DNA/MVA prime-boost HIV vaccine. Curr Opin Invest Drugs 2007; 8: 159–167.

107. Abaitua F, Rodriguez JR, Garzon A, et al. Improving recombinant MVA immune responses: potentiation of the immune responses to HIV-1 with MVA and DNA vectors expressing Env and the cytokines IL-12 and IFN-gamma. Virus Res 2006; 116: 11–20.

108. Taylor J, Tartaglia J, Riviere M, et al. Applications of canarypox (ALVAC) vectors in human and veterinary vaccination. Dev Biol Stand 1994; 82: 131–135.

109. Goepfert PA, Horton H, McElrath MJ, et al. High-dose recombinant Canarypox vaccine expressing HIV-1 protein, in seronegative human subjects. J Infect Dis 2005; 192: 1249–1259.

110. McConnell MJ, Imperiale MJ. Biology of adenovirus and its use as a vector for gene therapy. Hum Gene Ther 2004; 15: 1022–1033.

111. Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. Biotechnology 1992; 20: 363–390.

112. Schulick AH, Vassalli G, Dunn PF, et al. Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. Potential for immunosuppression and vector engineering to overcome barriers of immunity. J Clin Invest 1997; 99: 209–219.

113. Casimiro DR, Wang F, Schleif WA, et al. Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with DNA and recombinant adenoviral vaccine vectors expressing Gag. J Virol 2005; 79: 15547–15555.

114. Caley IJ, Betts MR, Davis NL, et al. Venezuelan equine encephalitis virus vectors expressing HIV-1 proteins: vector design strategies for improved vaccine efficacy. Vaccine 1999; 17: 3124–3135.

115. Perri S, Greer CE, Thudium K, et al. An alphavirus replicon particle chimera derived from Venezuelan equine encephalitis and sindbis viruses is a potent gene-based vaccine delivery vector. J Virol 2003; 77: 10394–10403.

116. Riezebos-Brilman A, de Mare A, Bungener L, et al. Recombinant alphaviruses as vectors for anti-tumour and anti-microbial immunotherapy. J Clin Virol 2006; 35: 233–243.

117. Sun JY, Anand-Jawa V, Chatterjee S, et al. Immune responses to adeno-associated virus and its recombinant vectors. Gene Ther 2003; 10: 964–976.