14

Vaccines

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INTRODUCTION

Since vaccination was documented by Edward Jenner in 1798, it has become the most successful means of preventing infectious diseases, saving millions of lives every year. Application of vaccines is currently not limited to the prevention of infectious diseases. Vaccines in the pipeline include, amongst others, therapeutic vaccines against allergies, cancer, and Alzheimer's disease.

Modern biotechnology has an enormous impact on current vaccine development. The elucidation of the molecular structures of pathogens and the tremendous progress made in immunology as well as developments in proteomics and bioinformatics have led to the identification of protective antigens and ways to deliver them. Together with technological advances, this has caused a move from empirical vaccine development to more rational approaches to develop effective and safe vaccines. In addition, modern methodologies may provide simpler and cheaper production processes for selected vaccine components.

Although vaccines resemble other biopharmaceuticals such as therapeutic proteins in some aspects, there are several important differences (Table 14.1). Unique features of vaccines include the low dose and frequency of administration, and the widely different vaccine categories (Table 14.2). Also, the target group is not only patients but basically every human being on the planet, with the emphasis on very young, healthy children. These differences have a huge impact on the requirements for vaccine admission on the market and release of vaccine batches, putting safety requirements on par with efficacy.

In the following section, immunological principles that are important for vaccine design are summarized. Subsequently, vaccine categories will be discussed, including current developments, especially in the field of therapeutic cancer vaccines. It is not our intent to provide a comprehensive review. Rather, we will explain current approaches to vaccine development and illustrate these approaches with representative examples. Routes of administration will be discussed in a separate section. In the last section, pharmaceutical aspects of vaccines, including issues related with production, formulation, characterization and storage, are dealt with.

IMMUNOLOGICAL PRINCIPLES

■ Introduction

As a reaction to infection, the human immune system launches a series of immunological responses with the goal of eliminating the pathogen. Innate immune cells will be the first to respond and will attempt to clear the pathogen through phagocytosis and/or lysis. As pathogens have developed strategies to evade the innate immune response, all vertebrates are capable of eliciting a highly specific response by virtue of their adaptive immune system. The adaptive immune system can generate humoral immunity and cell-mediated immunity (see Fig. 14.1 and Table 14.3). Antibodies, produced by B-cells, are the typical representatives of humoral immunity. An antibody belongs to one of four different immunoglobulin classes (IgM, IgG, IgA, or IgE) (cf. Chap. 8). Antibodies are able to prevent infection or disease through several mechanisms:

1. Binding of antibodies covers the pathogen with Fc (constant fragment), the "rear end" of immunoglobulins. Phagocytic cells, such as macrophages,

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Characteristic	Vaccines	Other biopharmaceuticals
Dose	Low (microgram range)	High (usually milligram range)
Frequency	Low (months –decades)	High (days – weeks)
Product group	Heterogeneous	Less heterogeneous
Characteristics	Sometimes ill-defined,	Mostly well-defined
Type of formulation	Usually a suspension or emulsion (liquid or lyophilized)	Usually a solution (liquid or lyophilized)
Indication	Mostly prophylactic	Therapeutic
Target group	Every human being	Patients
Number of active ingredients	>1 (antigen (s) and adjuvant(s))	1

Table 14.1 ■ Exemplary differences between vaccines and most other biopharmaceuticals

Vaccine (sub)type	Example(s)	Antigen	Disease	Prophylactic/ Therapeutic	Status of Development*
Live					
Attenuated viruses	Poliovirus (Sabin)	Whole attenuated virus	Polio	P	2
	Rotavirus		Diarrhea	P	2
	Measles virus		Measles	P	2
	Mumps virus		Mumps	P	2
	Varicella zoster		Chickenpox	P	2
	Yellow fever virus		Yellow fever	P	2
Attenuated bacteria	BCG	Whole attenuated bacteria	Tuberculosis	p	2
	Salmonella typhi		Typhoid fever	P	2
Vectored	HIV antigens using vaccinia or adenoviral vectors	Multiple HIV antigens	AIDS	P	1
	Ebola antigens using adenoviral or vesicular stomatitis virus vectors	cAd3-EBO Z, VSV ZEBOV	Ebola virus disease	P	1
Human cells	Autologous dendritic cells (Provenge)	Prostate acid phosphatase (PAP)	Prostate cancer	T	2

Table 14.2 ■ Vaccine categories based on antigen source

Vaccine (sub)type	Example(s)	Antigen	Disease	Prophylactic/ Therapeutic	Status of Development*
Inactivated					
Whole virus	Poliovirus (2ndgeneration)	Formaldehyde-inactivated poliovirus	Polio	P	2
	Rabies virus	Propriolactone-inactivated Pitman-Moore L503 rabies virus strain	Rabies	P	2
	Hepatitis A virus	Formaldehyde-inactivated Hepatitis A virus strain CR 326F antigen	Hepatitis	P	2
Whole bacteria	*Bordetella pertussis*	Heat or formaldehyde inactivated bacteria	Whooping cough	P	2
	Vibrio cholerae		Cholera	P	2
Human cells	Melacine	Melanoma cell extract	Melanoma	T	2
Subunits					
Proteins	Mosquirix	CSP-HBsAg fusion protein	Malaria	P	2
	Hepatitis B virus	HBsAg VLP	Hepatitis B	P	2
	Gardasil	HPV-6/11/16/18 VP1 VLPs	Cervical cancer	P	2
	Clostridium tetani	Tetanus toxoid	Tetanus	P	2
	Corynebacterium diphtheriae	Diphtheria toxoid	Diphtheria	P	2
Peptides	ISA101 synthetic long peptides (SLP)	HPV16 E6 and E7 T cell epitopes	Cervical cancer	T	1
Polysaccharides	*Haemophilis influenzae* type B (Hib)	Capsular polysaccharide	Invasive Hib disease	P	2
	Neisseria meningitidis	Capsular polysaccharides	Meningitis	P	2
	Streptococcus pneumoniae	Capsular polysaccharides, either free or conjugated to CRM197 diphtheria toxoid	Pneumonia, meningitis	P	2
Nucleic acids					
DNA	Influenza vaccine	H5N1, H1N1	Flu	P	1
	Human papilloma virus vaccine	HPV 16/18 fusion consensus antigens	Cervical cancer	T	1
	HIV vaccine	HIV-1 gag, env and pol	AIDS	T	1
RNA	Self-adjuvanted mRNA	PSA, PSCA, PSMA, STEAP1	Prostate cancer	T	1

∗ *1. Clinical evaluation, 2. Marketed product*

Abbreviations:

HIV: human immunodeficiency virus; HPV: human papillomavirus; cAd3 EBO-Z: Chimp adenovirus serotype 3-vectored Zaire ebolavirus antigens; VSV ZEBOV: Vesicular stomatitis virus-vectored Zaire ebolavirus antigens; CSP-HBsAg: circumsporozoite protein fused toHepatitis virus B surface antigen. VLP: virus-like particle; CRM197: genetically detoxified form of diphtheria toxin. H5N1, H1N1: influenza virus A subtypes, with differences in the surface antigens hemagglutinin (H) and Neuraminidase (N); HIV-1 gag, env and pol: the 3 major proteins of HIV-1, with gag being a polyprotein that is processed to matrix and core proteins, env a protein residing in the lipid envolope and pol the reverse transcriptase; PSA: prostate-specific antigen; PSCA: prostate stem cell antigen; PSMA: prostate-specific membrane antigen; STEAP1: prostate-specific metalloreductase
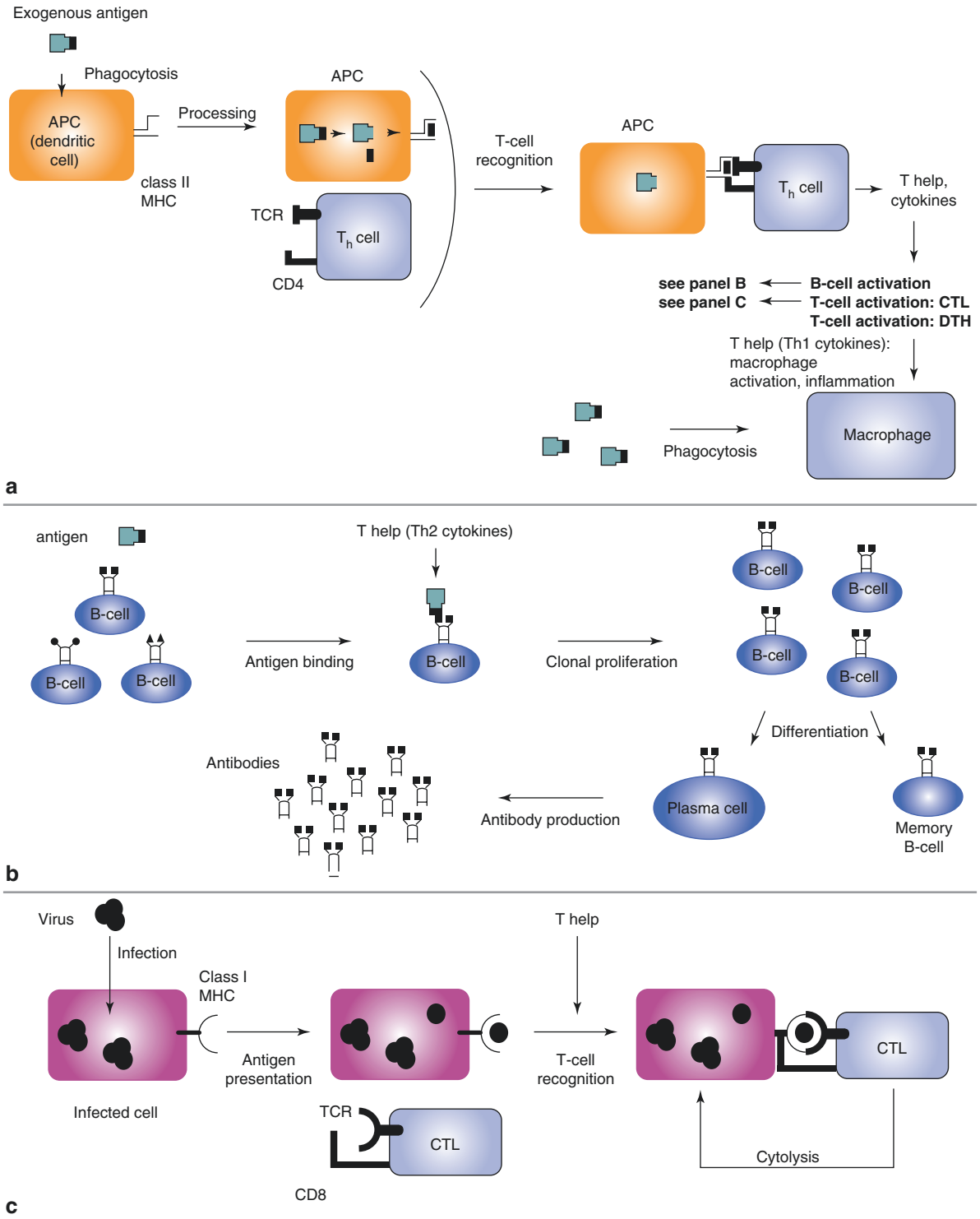
Table 14.2 ■ (continued)

Figure 14.1 ■ Schematic representation of antigen-dependent immune responses. (**a**) Activation of T-helper cells (Th-cells). An antigen-presenting cell (APC), e.g., a dendritic cell, phagocytoses exogenous antigens (bacteria or soluble antigens) and degrades them partially. Antigen fragments are presented by MHC class II molecules to a CD4-positive Th-cell; the MHC-antigen complex on the APC is recognized by the T-cell receptor (TCR) and CD4 molecules on the Th-cell. The APC-Th-cell interaction leads to activation of the Th-cell. The activated Th-cell produces cytokines, resulting in the activation of macrophages (Th1 help), B cells (Th2 help; panel **b**), or cytotoxic T cells (panel **c**). (**b**) Antibody production. The presence of antigen and Th2-type cytokines causes proliferation and differentiation of B cells. Only B cells specific for the antigen become activated. The B cells, now called plasma cells, produce and secrete large amounts of antibody. Some B cells differentiate into memory cells. (**c**) Activation of cytotoxic T lymphocytes (CTLs). CTLs recognize nonself antigens expressed by MHC class I molecules on the surface of virally infected cells or tumor cells. Cytolytic proteins are produced by the CTL upon interaction with the target cell

express surface receptors for Fc. This allows targeting of the opsonized (antibody-coated) antigen to these cells, followed by enhanced phagocytosis.
2. Immune complexes (i.e., complexes of antibodies bound to target antigens) can activate complement, a system of proteins which then becomes cytolytic to bacteria, enveloped viruses, or infected cells.
3. Phagocytic cells may express receptors for complement factors associated with immune complexes. Binding of these activated complement factors enhances phagocytosis.

Immune response	Immune product	Accessory factors	Infectious agents
Humoral	IgG	Complement, neutrophils	Bacteria and viruses
	IgA	Alternative complement pathway	Microorganisms causing respiratory and enteric infections
	IgM	Complement, macrophages	(Encapsulated) bacteria
	IgE	Mast cells	Extracellular parasites
Cell mediated	CTL	Cytolytic proteins	Viruses, mycobacteria, intracellular parasites
	Th1	Macrophages	Mycobacteria, treponema (syphilis), fungi

Table 14.3 ■ Important immune products protecting against infectious diseases

4. Viruses can be neutralized by antibodies through binding at or near receptor binding sites on the virus surface. This may prevent binding to and entry into the host cell.

Antibodies are effective against many, but not all infectious microorganisms. They may have limited value when pathogens occupy intracellular niches (such as intracellular bacteria and parasites), which are not easily reached by antibodies. In this case cell-mediated immunity is required to clear the infected cells. T-cells are the major representative of cell-mediated immunity and can clear infections by the following mechanisms:
1. Cytotoxic T-lymphocytes (CTLs, also called cytotoxic T-cells) react with target cells and kill them by release of cytolytic proteins like perforin.
2. T-helper cells (Th1-type, see below) activate macrophages, allowing them to kill intracellular pathogens.

In contrast to the innate response, the adaptive immune response is very specific to the invading pathogen (Fig. 14.2). The adaptive immune system comprises B-cells and T-cells with a wide range of specificities, owing to the unique compositions of their B-cell receptor (BCR) and T-cell receptor (TCR). During an infection the innate immune system instructs those B- and T-cells that have BCRs and TCRs specific for the invading pathogen to proliferate and gain effector functions. When the infection is cleared, most of these B- and T-cells are obsolete and many will die by apoptosis. Antibodies produced by B-cells, however, can persist in the circulation for an extended period of time. Moreover, some of the B- and T-cells resist apop-
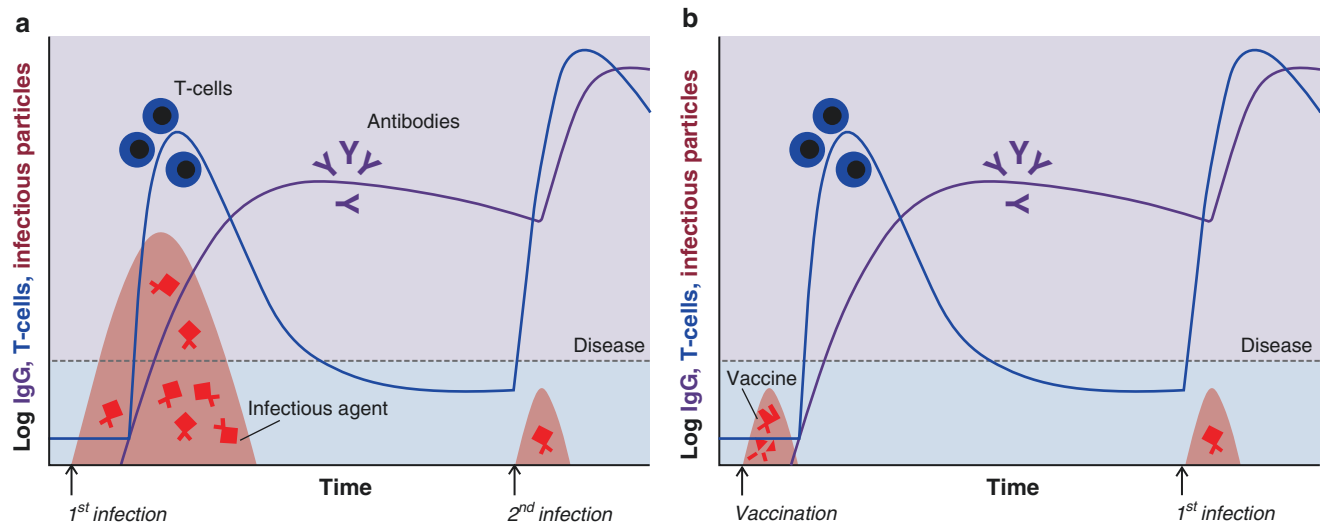

Figure 14.2 ■ Principle of adaptive immune responses following infection and vaccination. (**a**) Schematic representation of adaptive immune responses upon primary and secondary infection. Upon primary infection T- and B-cell responses take time to develop, allowing pathogens to proliferate and cause disease. Upon secondary infection, circulating antibodies and memory T-cells quickly respond, preventing proliferation and dissemination of the pathogen. (**b**) Application of a vaccine that induces an adaptive immune response like a natural infection, but without associated disease

tosis and can maintain themselves for many years as memory B- and T-cells. In contrast to their naive counterparts, these memory cells are rapidly activated and clonally expanded when they re-encounter the same pathogen on a later occasion. Therefore, unlike the primary response, the response after repeated infection is very fast and usually sufficiently strong to prevent reoccurrence of the disease (Fig. 14.2a).

Vaccination exploits the formation of this immunological memory by the adaptive immune system. The principle of vaccination is mimicking an infection in such a way that the natural specific defense mechanism of the host against the pathogen will be activated and immunological memory is established, but the host will remain free of the disease that normally results from a natural infection (Fig. 14.2b). This is effectuated by administration of antigenic components that consist of, are derived from, or are related to the pathogen. The immune response is highly specific: it discriminates not only between pathogen species but often also between different strains within one species (e.g., strains of meningococci, poliovirus, influenza virus). Albeit sometimes a hurdle for vaccine developers, this high specificity of the immune system allows an almost perfect balance between responsiveness to foreign antigens and tolerance to self-antigens.

Whereas prophylactic vaccines aim for immunological memory, the primary goal of therapeutic vaccines usually is induction of potent effector responses rather than memory. In the next paragraphs we will discuss the immunological principles leading to effector and memory responses.

■ Generation of an Immune Response and Immunological Memory

The generation of an immune response by vaccination follows several distinct steps that should ultimately lead to a potent effector response and/ or long-lasting memory. After administration of the vaccine, the first step is uptake by professional antigen-presenting cells (APCs) at the site of application. APCs are able to shuttle the vaccine components to secondary lymphoid organs and present the antigens to T- and B-lymphocytes, which—under the right conditions—results in activation of these lymphocytes. This simplified process is illustrated in Fig. 14.3. Below we describe in more detail the successive steps leading to an immune response, in particular the steps relevant for the design of vaccines.

Activation of the Innate Immune System
Every immune reaction against a pathogen or a vaccine starts with activation of the innate immune system. Although the innate response itself does not lead to immunological memory, it is instrumental in activating and educating the adaptive immune system. Important constituents of the innate immune system are APCs like macrophages and dendritic cells (DCs), which reside in tissues. By continuously endocytosing extracellular material, they sample their environment for potential harmful materials. To distinguish harmful from innocuous substances, APCs are equipped with pattern recognition receptors (PRRs) that allow detection of conserved microbial and viral structures, called pathogen-associated molecular patterns (PAMPs) (Kawai and Akira 2009). Examples of PAMPs are viral RNA and bacterial cell wall constituents, such as lipopolysaccharide (LPS) and flagellin (Table 14.4). As pathogens occupy different cellular niches, PRRs can be found either on the cell surface and endosomes (for bacterial PAMPs) or in the cytoplasm (for viral PAMPs). Examples of PRRs are toll-like receptors (TLRs), C-type lectins and RIG-I-like receptors (Table 14.4).

PRR activation induces a maturation program, which switches APCs from an antigen sampling to an antigen presentation mode, which is critical for their role as intermediates for lymphocyte activation. PRR activation induces expression of MHC class I (MHCI) and MHC class II (MHCII) molecules, increasing the APCs' capacity to present antigen to T-cells. Moreover, APCs will gain expression of chemokine receptors (e.g., CCR7) that allow them to migrate to secondary lymphoid tissue. Finally, PRR stimulation induces upregulation of co-stimulatory molecules and pro-inflammatory cytokines, which provide import activation signals to T-cells during antigen presentation.

PRR activation is an essential step in the vaccination process and therefore important to consider when designing a vaccine. Live attenuated or inactivated vaccines naturally contain PAMPs to activate PRRs, however subunit vaccines may lack these PAMPs and may require addition of adjuvants (see section "Formulation").

Antigen Presentation
The peripheral lymphoid organs are the primary meeting place between cells of the innate immune system (APCs) and cells of the adaptive immune system (T-cells and B-cells). Whereas APCs are distributed throughout peripheral tissues, T- and B-cells are primarily located in secondary lymphoid organs, such as lymph nodes, spleen and Peyer's patches. An important reason for this is that, although the human body harbors a large number of lymphocytes (ca. 10^{12}), only few T- and B-cells will have a TCR or BCR that is specific for the antigen of interest. By concentrating lymphocytes in secondary lymphoid organs and having APCs presenting antigen there, the
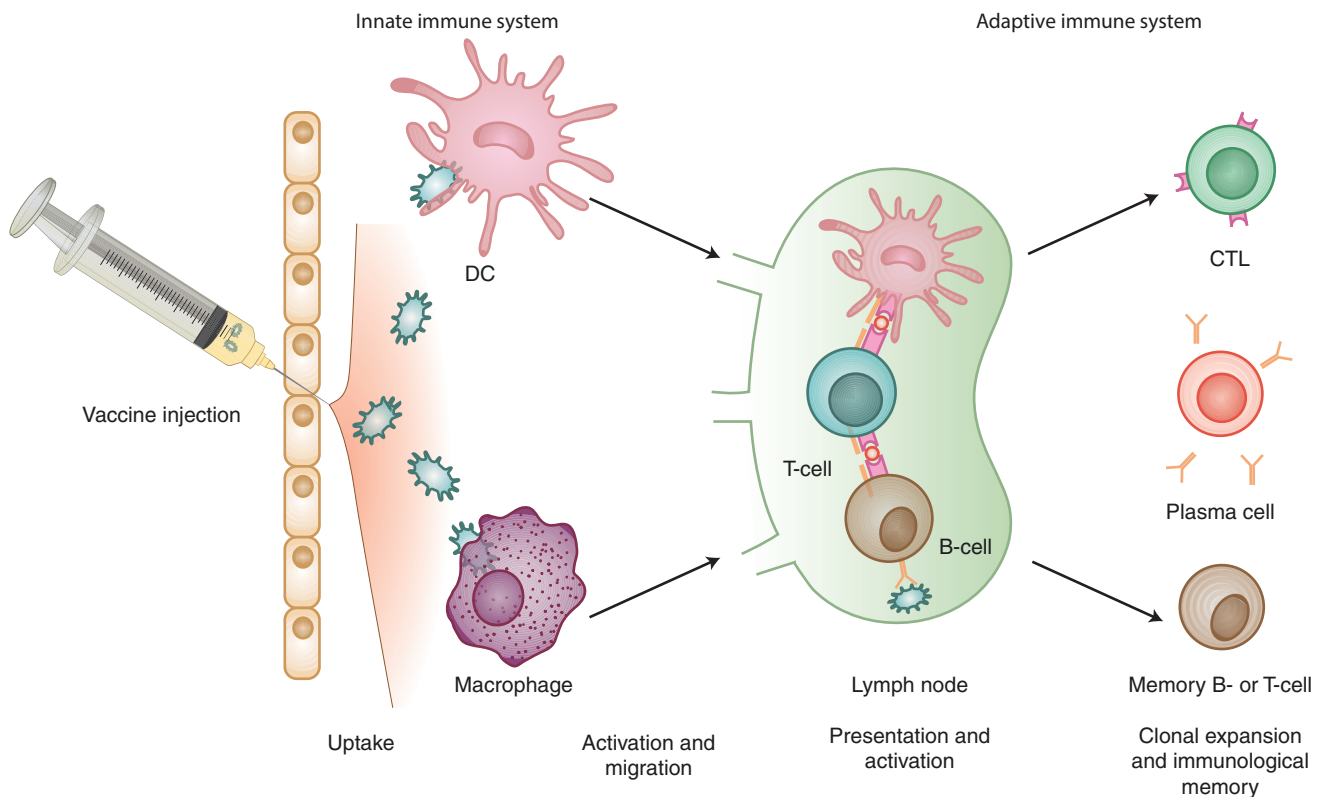
Figure 14.3 ■ Overview of the steps leading to immunity after administration of a vaccine. Upon subcutaneous or intramuscular administration, the vaccine components are taken up by phagocytic cells such as macrophages and dendritic cells (DCs) that reside in the peripheral tissue and express pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Professional antigen-presenting cells (APCs) that have taken up antigens become activated and start migrating towards nearby lymph nodes. Inside the lymph nodes, the antigen processed by the APCs is presented to lymphocytes, which, when recognizing the antigen and receiving the appropriate co-stimulatory signals, become activated. These antigen-specific B- and T-cells clonally expand to produce multiple progenitors recognizing the same antigen. In addition, memory B- and T-cells are formed that provide long-term (sometimes lifelong) protection against infection with the pathogen

chance of antigen specific T- and B-cells encountering their cognate antigen is increased. Upon interaction with APCs, antigen specific T-cells and B-cells will be activated, provided that they acquire the appropriate signals.

The first of these signals is antigen presentation, which allows selection of antigen specific B- and T-cells. B-cells and T-cells recognize antigens in different ways. B-cells can recognize antigens in their native form as their BCR allows direct interaction with the antigen. Therefore, B-cell antigens do not require major processing. In fact, B-cells can take up antigens that are small enough to drain to lymph nodes without the help of APCs. To shuttle larger antigens to lymphoid tissue in their native form, APCs express receptors that allow presentation of intact antigens to B-cells (Batista and Harwood 2009).

Some antigens are able to directly stimulate antibody production by B-cells without T-cell involvement. These thymus-independent antigens include certain linear antigens that are not readily degraded in the body and have a repeating determinant, such as bacterial polysaccharides. Thymus-independent antigens do not induce immunological memory and are therefore less interesting from a vaccination standpoint. It is possible, however, to render these antigens thymus dependent by chemically coupling them to a protein carrier (see sections "B-cell and T-cell Activation" and "Polysaccharide Vaccines").

T-cells are unable to directly interact with antigen, but depend on the APCs to process antigens into peptide fragments (T-cell epitopes) and present them to the T-cells in the context of MHCI (to CD8+ T-cells) or MHCII molecules (CD4+ T-cells) on the APC surface. Whether antigens are presented on MHCI or MHCII molecules is dependent on the intracellular location of the antigen processing. Exogenous antigens, acquired by endocytosis, can undergo limited proteolysis in the endosome and associate with MHCII molecules (Fig. 14.1a). Loaded MHCII molecules return to the surface and can

PRR	PRR location	PAMP[b]	Source
TLR1–TLR2	Cell surface	Triacyl lipopeptides	Bacteria
TLR2–TLR6	Cell surface	Diacyl lipopeptides	Bacteria
		Zymosan	Fungus
TLR3	Endosome	dsRNA	Virus
TLR4	Cell surface	LPS	Bacteria
TLR5	Cell surface	Flagellin	Bacteria
TLR7	Endosome	Single stranded (ss) RNA	RNA viruses
TLR8	Endosome	ssRNA	RNA viruses
TLR9	Endosome	CpG DNA	Bacteria
RIG-I	Cytosol	ssRNA and short double stranded RNA	Viruses
MDA5	Cytosol	Long dsRNA	Viruses
LGP2 (helicase)	Cytosol	RNA	Viruses
NOD1/ NLRC1	Cytosol	iE-DAP	Bacteria
NOD2/ NLRC2	Cytosol	MDP	Bacteria
		ATP	Bacteria/ host
		Uric acid, CPPD, amyloid-β	Host
NALP1/ NLRP1	Cytosol	Anthrax lethal toxin	Bacteria
IPAF/NLRC4	Cytosol	Flagellin	Bacteria
NAIP5	Cytosol	Flagellin	Bacteria
Dectin-1	Cell surface	β-Glucan	Fungi

[a]Adapted from Kawai and Akira, Int. Immunol. 2009
[b]Several of the PAMPs listed here are used as vaccine adjuvants (see section "Formulation" and Table 14.6)

Table 14.4 ■ Examples of pattern recognition receptors (PRRs), their ligands (PAMPs) and source[a]

interact with CD4+ T-helper cells. Endogenous antigens, such as viral or mutated proteins produced by the host cell, are generated by proteasomal processing in the cytosol. The resulting peptides can associate with MHCI in the endoplasmatic reticulum and can interact with (CD8+) cytotoxic T-cells (Fig. 14.1c).

These different antigen presentation pathways have consequences for vaccine design. As T-cells only recognize processed antigen fragments, T-cell responses rely on continuous epitopes, which are linear peptide sequences (usually consisting of up to ten amino acid residues) of the protein (see Fig. 14.1a). In contrast, B-cell epitopes can be discontinuous epitopes comprising amino acid residues sometimes far apart in the primary sequence, which are brought together through the unique folding of the protein (see Fig. 14.1b). Antibody recognition of B-cell epitopes, whether continuous or discontinuous, is usually dependent on the conformation (=three-dimensional structure) of the antigen. For vaccines aimed to induce high levels of neutralizing antibodies (for instance, diphtheria and tetanus vaccines), one should take great care that the antigen remains in its native form. Vaccines that should induce CTL responses (e.g., some virus vaccines, cancer vaccines) will not necessarily require the antigen to be in its native form, as the antigen will have to be degraded before presentation anyway. A major challenge for these types of vaccines, however, is that MHCI presentation requires antigen to enter the cytosol rather than the endosomal compartment of an APC (see Fig. 14.1c). Professional APCs, especially DCs, have the capacity to transfer exogenously acquired antigens from the endosomal compartment into the MHCI processing pathway. The process is referred to as cross-presentation.

B-cell and T-cell Activation

Next to TCR stimulation through peptide loaded MHCI or MHCII molecules, the second signal T-cells require is co-stimulation via interaction of accessory and co-stimulatory molecules on the APCs (Fig. 14.4). This cell-cell interaction is essential for proper stimulation of lymphocytes, and without those accessory signals, antigen-specific T-cells will not proliferate and may become anergic (i.e., acquire a state of unresponsiveness). As co-stimulatory molecules, such as CD80/86, CD40 and ICAM-1, are upregulated on APCs after PRR stimulation, this signal functions as an additional safety check to prevent unwanted immune responses against self-antigens.

T-cells receiving TCR stimulation and co-stimulation will become activated, clonally expand and generate multiple progenitors all recognizing the same antigen. In contrast, T-cells can also receive co-inhibitory signals from the APC (e.g., PD1, CTLA4 activation). These signals reduce T-cell activation and provide a negative control mechanism against uncontrolled or unwanted T-cell responses.

Before and during clonal expansion, T-cells receive cytokine signals that influence their fate (signal 3). Cytokines can promote T-cell proliferation and also affect their effector function (See Fig. 14.4). For instance, interleukin 12 (IL-12) and type I interferon (IFN) are cytokines that are essential for the development of CTLs. Lack of these cytokines results in reduced proliferation of CTLs and a reduced capacity to kill target cells.
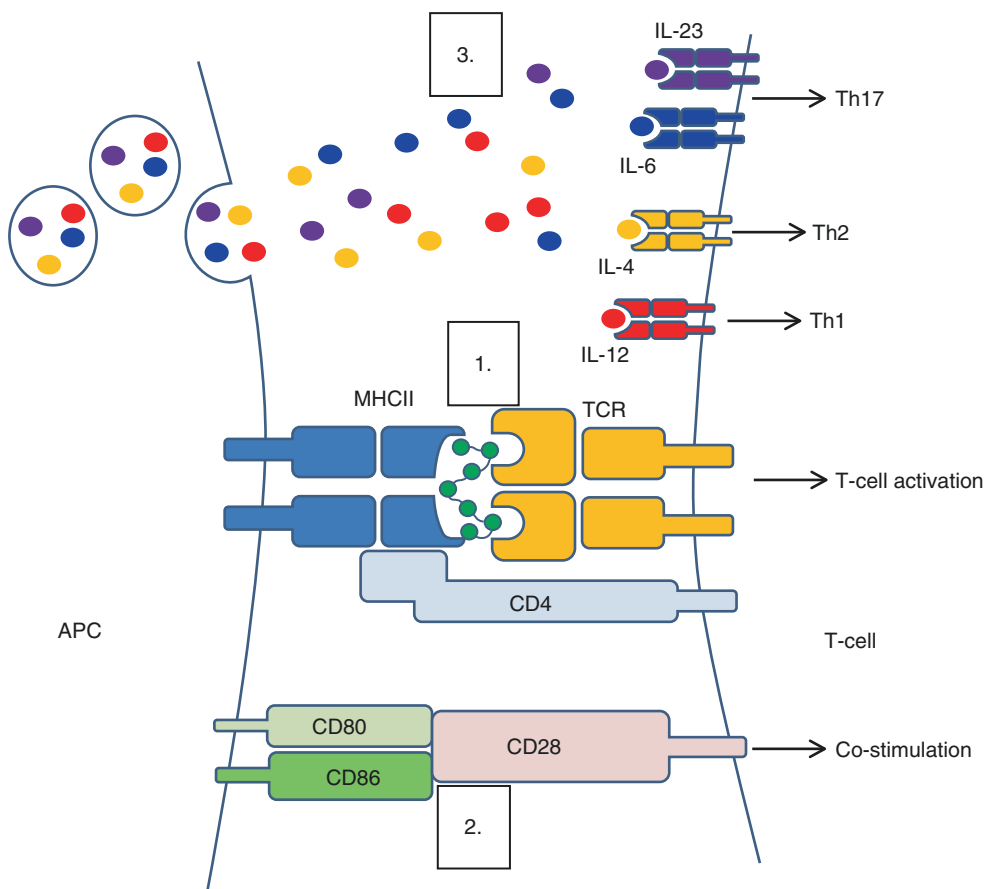
Figure 14.4 ■ The 3 signals of T-helper cell activation. 1. Antigen presentation. Peptides derived from a vaccine are loaded on MHCII molecules by the APC and presented to the T-cell receptor (TCR) on T-cells. 2. Co-stimulation. Activated APCs express co-stimulatory molecules, such as CD80/86 which support T-cell activation through interaction with CD28 on T-cells. 3. Cytokines. APCs can produce different cytokines depending on the type of PAMP that has activated the APC. These cytokines provide a third signal to the T-cell by engaging their cognate receptors on the T-cell surface. Whereas IL-12 (red) signaling leads to Th1 polarization of the CD4+ T-cell, IL-4 (yellow) signaling induces Th2 polarization and IL-6/IL23 (blue/purple) signaling provides a pathway towards Th17 CD4+ T-cells

Especially for the CD4+ T-helper cells the cytokine signal during priming is crucial, as T-helper cells can have various effector functions. For instance, in the presence of cytokines, such as IL-12 and type I IFN, CD4+ T-cells develop into T-helper 1 (Th1) cells. These cells produce cytokines, such as IFN-γ and tumor necrosis factor alfa (TNF-α), which potentiate the effector function of phagocytes and increase inflammation. Therefore, induction of memory Th1 cells is a major goal for vaccines that aim to protect against intracellular pathogens. T-helper 2 (Th2) cells develop under influence of IL-4 signaling. These Th2 cells produce another set of cytokines that prevent Th1 differentiation and support B-cell proliferation and differentiation. Th2 cells have therefore been associated with increased humoral responses. However, as the cytokines produced by Th2 cells have been linked to IgE production by B-cells, reducing the number of memory Th2 cells has become an important focus in the design of vaccines aiming to reduce allergic responses.

Next to Th1 and Th2 cells, various other T-helper subsets have been identified, each having unique functional properties. Th17 cells develop when CD4+ T-cells receive transforming growth factor beta (TGF-β), IL-6 and IL-23 signals, and produce IL-17 and IL-22. These cytokines support the defense of mucosal surfaces, but have also been linked to inflammatory disease, such as inflammatory bowel disease and psoriasis. Regulatory T-cells (Tregs) are subsets of CD4+ T-cells that play an important role in limiting inflammation through secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β. Induction of Tregs may be of interest for vaccines that aim to reduce inflammation in autoimmune diseases.

One particular subset of Th-cells is devoted to providing help to B-cells, the so-called T-follicular helper cells (Tfhs). Under influence of IL-6 and IL-21, Tfhs upregulate molecules, such as C-X-C chemokine receptor type 5 (CXCR5), allowing them to migrate into B-cell zones. There, Tfhs can interact with B-cells that present cognate antigen on MHCII molecules. Only B-cells that receive co-stimulatory signals from Tfhs will be able to generate high-affinity IgG antibodies or mature into memory B-cells. This can have consequences for vaccine design, as vaccines that are aimed to generate B-cell memory need to contain both B-cell and T-cell epitopes in one entity. For instance, polysaccharides derived from *Haemophilus influenzae* type b, *Neisseria meningitidis*, or *Streptococcus pneumoniae* are targets for neutralizing antibodies, but require conju-
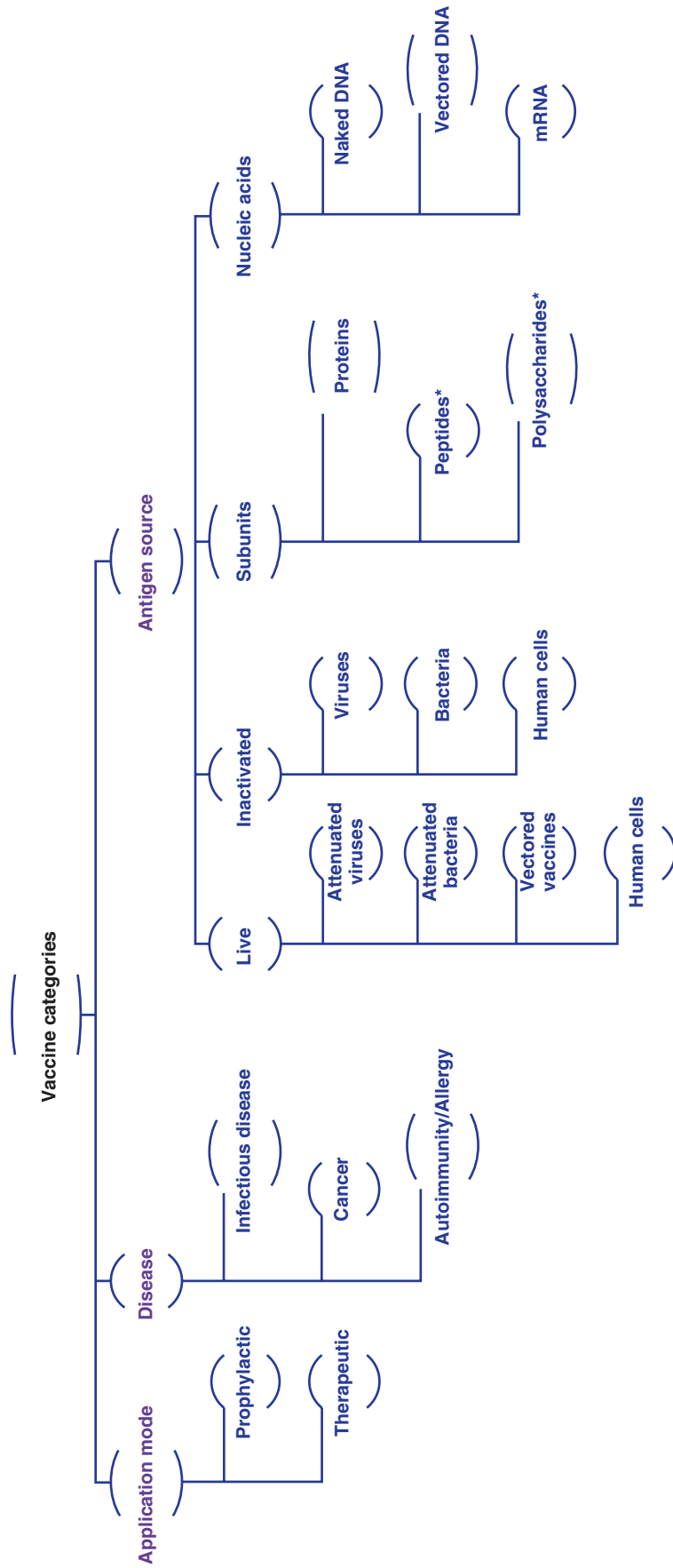
Figure 14.5 ■ Vaccine categories based on type of treatment, type of disease and antigen source

gation to a protein to allow T-cell help and development of B-cell memory.

VACCINE CATEGORIES

Vaccines can be classified based on whether they are aimed to prevent (prophylactic) or cure (therapeutic) a disease, the type of disease to treat (infectious diseases, allergy, autoimmune disease, cancer, etc.), or the antigen source used for vaccination (e.g., whole pathogens, subunits, peptides, or nucleic acids), as illustrated in Fig. 14.5. Below we first discuss vaccine categories based on antigen source. Next, current developments on therapeutic vaccines against cancer and other diseases are highlighted.

■ Classification Based on Antigen Source

Traditional vaccines originate from viruses or bacteria and can be divided in vaccines consisting of live attenuated pathogens and nonliving (inactivated) pathogens. In case the antigens that can convey immunity are known, specific subunits derived from the pathogen, such as proteins or polysaccharides, can be formulated into a vaccine. Nowadays such subunit vaccines can also be made recombinantly (in case of proteins), or by chemical conjugation to a carrier protein (in case of polysaccharides) to enhance the immune response to the antigenic components. Moreover, with our current knowledge on immune recognition, both B- and T-cell epitopes can be identified and synthetically made. Finally, nucleic acids form a separate class of antigen source, in which the DNA or RNA encoding the antigen(s) of interest is transfected into host cells to enable endogenous production and presentation of protein antigens. An overview of the various categories of vaccines and examples thereof is given in Table 14.2 and will be detailed in the sections below.

Live Attenuated Vaccines
Before the introduction of recombinant DNA (rDNA) technology, live vaccines were made by the attenuation of virulent microorganisms by serial passage and selection of mutant strains with reduced virulence or toxicity. Examples are vaccine strains for current vaccines such as oral polio vaccine, measles-mumps-rubella (MMR) combination vaccine, yellow fever vaccine and tuberculosis vaccine consisting of bacille Calmette-Guérin (BCG). An alternative approach is chemical mutagenesis. For instance, by treating *Salmonella typhi* with nitrosoguanidine, a mutant strain lacking some enzymes that are responsible for the virulence was isolated (Germanier and Fuer 1975).

Live attenuated vaccines have the advantage that after administration they may replicate in the host, similar to their pathogenic counterparts. This confronts the host with a larger and more sustained dose of antigen and PAMPs, which means that few and low doses

are required. In general, the vaccines give long-lasting humoral and cell-mediated immunity.

Live attenuated vaccines also have drawbacks. Live viral vaccines bear the risk to revert to a virulent form, although this is unlikely when the attenuated seed strain contains several mutations. Nevertheless, for diseases such as viral hepatitis, AIDS and cancers, this drawback makes the use of traditional live vaccines virtually unthinkable. Furthermore, it is important to recognize that immunization of immune-deficient children or immunocompromised adults with live organisms can lead to serious complications. For instance, a child with T-cell deficiency may become overwhelmed with BCG and die. Similarly, patients using certain immunosuppressive drugs (e.g., cyclosporin, methotrexate) should not be vaccinated with live attenuated vaccines.

Genetically Attenuated Live Vaccines
Emerging insights in molecular pathogenesis of many infectious diseases make it possible to attenuate microorganisms more efficiently nowadays. By making multiple deletions, the risk of reversion to a virulent state during production or after administration can be virtually eliminated. A prerequisite for attenuation by genetic engineering is that the factors responsible for virulence and the life cycle of the pathogen are known in detail. It is also obvious that the protective antigens or epitopes must be known: attenuation must not result in reduced immunogenicity.

An example of an improved live vaccine obtained by homologous genetic engineering is the oral cholera vaccine Vaxchora. An effective cholera vaccine should induce a local, humoral response in order to prevent colonization of the small intestine. Initial trials with *Vibrio cholerae* cholera toxin (CT) mutants caused mild diarrhea, which was thought to be caused by the expression of accessory toxins. A natural mutant was isolated that was negative for these toxins. Next, CT was detoxified by rDNA technology. The resulting vaccine strain, called CVD 103, is well tolerated and challenge experiments with adult volunteers showed protection (Levine et al. 2017; Garcia et al. 2005).

Genetically attenuated live vaccines have the general drawbacks mentioned in the paragraph about classically attenuated live vaccines. For these reasons, it is not surprising that homologous engineering is mainly restricted to pathogens that are used as starting materials for the production of subunit vaccines (see the section "Subunit Vaccines," below).

Live Vectored Vaccines
A way to improve the safety or efficacy of vaccines is to use live, avirulent, or attenuated bacteria or viruses as a carrier to express protective antigens from a patho-
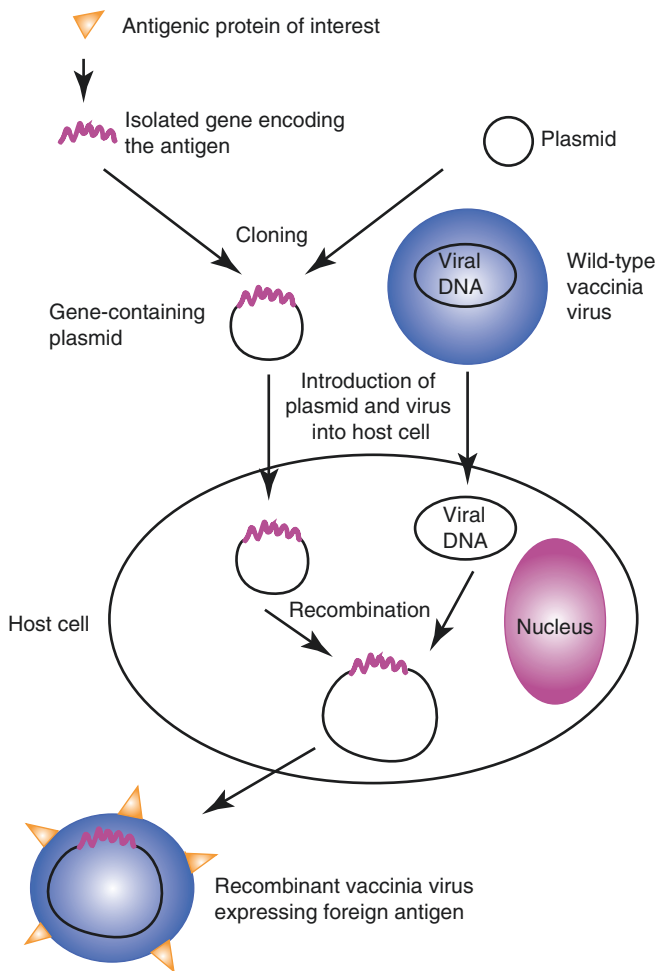
Figure 14.6 ■ Construction of recombinant vaccinia virus as a vector of foreign protein antigens. The gene of interest encoding an immunogenic protein is inserted into a plasmid. The plasmid containing the protein gene and wild-type vaccinia virus are then simultaneously introduced into a host cell line to undergo recombination of viral and plasmid DNA, after which the foreign protein is expressed by the recombinant virus

gen (see Table 14.2 for examples). Live vectored vaccines are created by recombinant technology, wherein one or more genes of the vector organism are replaced by one or more protective genes from the pathogen. Administration of such live vectored vaccines results in efficient and prolonged expression of the antigen-encoding genes either by the vaccinated individual's own cells or by the vector organism itself (e.g., in case of a bacterial vector).

Most experience has been acquired with vaccinia virus by using the principle that is schematically shown in Fig. 14.6. Advantages of vaccinia virus as vector include (1) its proven safety in humans as a smallpox vaccine, (2) the possibility for multiple immunogen expression, (3) the ease of production, (4) its relative heat resistance, and (5) its various possible administration routes. A multitude of live recombinant

vaccinia vaccines with viral and tumor antigens have been constructed, several of which have been tested in the clinic (Njuguna et al. 2014; Buchbinder et al. 2017; Payne et al. 2017). It has been demonstrated that the products of genes coding for viral envelope proteins can be correctly processed and inserted into the plasma membrane of infected cells.

Adenoviruses can also be used as vaccine vectors (see also Chap. 16). Adenoviruses have several characteristics that make them suitable as vaccine vectors: (1) they can infect a broad range of both dividing and non-dividing mammalian cells, which expands possibilities to select production cell lines; (2) transgene expression is generally high and can be further increased by using heterologous promoter sequences; (3) adenovirus vectors are mostly replication deficient and do not integrate their genomes into the chromosomes of host cells, making these vectors very safe to use; and (4) upon parenteral administration, adenovirus vectors induce strong immunity and evoke both humoral and cellular responses against the expressed antigen. A number of clinical trials with human or chimpanzee adenovirus vectors (HAd5, ChAd3) expressing antigens of Ebola virus, human immunodeficiency virus (HIV), and severe acute respiratory syndrome (SARS) have been performed (Cohen and Frahm 2017).

A major limitation of the use of live vectored vaccines is the prevalence of preexisting immunity against the vector itself, which could neutralize the vaccine before the immune system can be primed. Such preexisting immunity has been described for adenoviral vectors, for which the prevalence of neutralizing antibodies can be as high as 90% of the total population. The use of human or nonhuman (e.g., chimpanzee) strains with no or low prevalence of preexisting immunity as live vectors is therefore recommended (Ahi et al. 2011; Wong et al. 2018).

Inactivated Vaccines

An early approach for preparing vaccines is the inactivation of whole bacteria or viruses. A number of chemical reagents (e.g., formaldehyde, glutaraldehyde, β-propriolactone) and heat are commonly used for inactivation. Examples of inactivated vaccines are whole cell pertussis, cholera, typhoid fever, and polio vaccines. Inactivation may result in the loss of relevant epitopes due to covalent changes or partial unfolding of antigens. Also, since these vaccines do not replicate in vivo, often a higher dose is needed to induce protection, as compared to live attenuated vaccines. This may increase the price.

Subunit Vaccines

Given the complexity and batch-to-batch variability of vaccines consisting of inactivated whole pathogens,

the use of well-defined antigenic subunits of pathogens is desired. Such subunits can be antigens (proteins or polysaccharides) directly purified from the pathogen, recombinantly produced protein antigens, or synthetic peptides.

Diphtheria Toxoid and Tetanus Toxoid Vaccines

Some bacteria such as *Corynebacterium diphtheriae* and *Clostridium tetani* form toxins. Antibody-mediated immunity to the toxins is the main protection mechanism against the adverse effects of infections with these bacteria. Both toxins are proteins and are inactivated with formaldehyde for inclusion in vaccines. The immunogenicity of such toxoids is relatively low and is improved by adsorption of the toxoids to colloidal aluminum salts. This combination of an antigen and an adjuvant is still used in combination vaccines.

Polysaccharide Vaccines

Bacterial capsular polysaccharides consist of pathogen-specific multiple repeating carbohydrate epitopes, which are isolated from cultures of the pathogenic species. Plain capsular polysaccharides are thymus-independent antigens that are poorly immunogenic in infants and show poor immunological memory when applied in older children and adults. The immunogenicity of polysaccharides is highly increased when they are chemically coupled to carrier proteins containing T-cell epitopes. This coupling makes them T-cell dependent, which is due to the participation of Th-cells that are activated during the response to the carrier. Examples of such polysaccharide conjugate vaccines include meningococcal type C, pneumococcal, and *Haemophilus influenzae* type b (Hib) polysaccharide vaccines that are included in many national immunization programs.

Acellular Pertussis Vaccines

The relatively frequent occurrence of side effects of whole cell pertussis vaccine was the main reason to develop subunit pertussis vaccines. The development of such acellular pertussis vaccines in the 1980s exemplifies how a better insight into factors that are important for pathogenesis and immunogenicity can lead to improved vaccines: it was conceived that a subunit vaccine consisting of a limited number of purified immunogenic components and devoid of (toxic) bacterial LPS would significantly reduce undesired effects. Current licensed acellular pertussis vaccines contain one to four protein antigens. Although these vaccines are effective, they cannot prevent regular epidemics of whooping cough in many western countries. Short-lived immunity and vaccine induced selection of circulating strains resisting the primed immune system may contribute to this. Therefore, attempts are made to improve vaccination schemes and to develop new pertussis vaccines.

Recombinant Subunit Vaccines

To improve the yield, facilitate the production, and/or improve the safety of protein-based vaccines, protein antigens are nowadays often produced recombinantly, i.e., expressed by host cells that are safe to handle and/or allow high expression levels.

Heterologous hosts used for the expression of protein antigens include yeasts, bacteria, insect cells, plant cells, and mammalian cell lines. Hepatitis B surface antigen (HBsAg), which previously was obtained from plasma of infected individuals, has been expressed in baker's yeast, *Saccharomyces cerevisiae* (Vanlandschoot et al. 2002), and in mammalian cells, such as Chinese hamster ovary cells (Raz et al. 2001), by transforming the host cell with a plasmid containing the HBsAg-encoding gene. Both expression systems yield 22-nm HBsAg particles (also called virus-like particles or VLPs) that are structurally identical to the native virus. Advantages are safety, consistent quality, and high yields. The yeast-derived vaccine has become available worldwide and appears to be as safe and efficacious as the classical plasma-derived vaccine.

The two human papillomavirus (HPV) vaccines currently on the market are produced as recombinant proteins which, like HBsAg, assemble spontaneously into virus-like particles. Antigens for Gardasil, a quadrivalent HPV vaccine, are produced in yeast, whereas antigens for the bivalent vaccine Cervarix are produced in insect cells.

Recombinant Peptide Vaccines

After identification of a protective epitope, it is possible to incorporate the corresponding peptide sequence through genetic fusion into a carrier protein, such as HBsAg, hepatitis B core antigen, and β-galactosidase (Francis and Larche 2005). The peptide-encoding DNA sequence is synthesized and inserted into the carrier protein gene. An example of the recombinant peptide approach is a malaria vaccine based on a 16-fold repeat of the Asn-Ala-Asn-Pro sequence of a *Plasmodium falciparum* surface antigen. The gene encoding this peptide was fused with the HBsAg gene, and the fusion product was expressed by yeast cells (Vreden et al. 1991). Clinical trials with this candidate malaria vaccine demonstrated moderate efficacy in children and infants in Africa (RTS,S Clinical Trials Partnership 2015).

Genetic fusion of peptides with proteins offers the possibility to produce protective epitopes of toxic antigens derived from pathogenic species as part of nontoxic proteins expressed by harmless species. Furthermore, a uniform product is obtained in com-

parison with the variability of chemical conjugates (see the section "Synthetic Peptide Vaccines", below).

Synthetic Peptide Vaccines

In principle, a vaccine could consist of only the relevant epitopes instead of intact pathogens or proteins. Peptide epitopes are small enough to be produced synthetically and a peptide-based vaccine would be much better defined than traditional vaccines, making the concept of peptide vaccines attractive. However, it turned out to be difficult to develop these vaccines, and today there are no licensed peptide-based vaccines available yet. Nevertheless, important progress has been made, and some synthetic peptide vaccines have now entered the clinic, e.g., for immunotherapy of cancer (Melief and van der Burg 2008; van Poelgeest et al. 2013). To understand the complexity of peptide vaccines, one has to distinguish the different types of epitopes.

B-cell Epitope-Based Peptide Vaccines Epitopes recognized by antibodies or B-cells are very often conformation dependent (see above, section "Immunological Principles", and Van Regenmortel 2009). For this reason, it is difficult to identify them accurately and to synthesize them in the correct conformation. Manipulation of the antigen, such as digestion or the cloning of parts of the gene, will often affect B-cell epitope integrity. An accurate way of identifying epitopes is to elucidate the crystal structure of antigen-antibody complexes. This is difficult and time consuming, and although crystallography can reveal molecular interactions with unsurpassed detail, the molecular complex likely is much more dynamic in solution. Once the epitope is identified, synthesizing it as a functional peptide has proven to be difficult as well. The peptides need to be conformationally restrained. This can be achieved by cyclization of the peptide (Oomen et al. 2005) or by the use of scaffolds to synthesize complex peptide structures (Timmerman et al. 2007).

T-cell Epitope-Based Peptide Vaccines Regarding conformation, T-cell epitopes are less demanding because they are presented naturally as processed peptides by APCs to T-cells. As a result, T-cell epitopes are linear. Here, we discern CD8+ epitopes (8–10 amino acid residues; MHC class I restricted) and CD4+ epitopes (>12 amino acid residues; MHC class II restricted). The main requirement is that they fit into binding grooves of MHC molecules with high enough affinity. Studies with peptide-based cancer vaccines have shown that these should contain both CD8+ and CD4+ epitopes in order to elicit a protective immune response. Furthermore, minimal peptides that can be externally loaded on MHC molecules of cells have been shown to induce less robust responses than longer peptides that require intracellular processing after uptake by DCs. Another point to consider is the variable repertoire of MHC molecules in a patient population, implying that a T-cell epitope-based peptide vaccine should contain several T-cell epitopes in order to be effective in the majority of the vaccinated population. Following these concepts, clinical trials with overlapping long peptide vaccines have shown promising results in the immunotherapy of patients with HPV-induced malignancies (Melief and van der Burg 2008).

Nucleic Acid Vaccines

Immunization with nucleic acid vaccines involves the administration of genetic material, plasmid DNA or messenger RNA (mRNA), encoding the desired antigen. The encoded antigen is then expressed by the host cells and after which an immune response against the expressed antigen is raised. Nucleic acid vaccines offer the safety of subunit vaccines and the advantages of live recombinant vaccines. They can induce strong CTL responses against the encoded antigen. In addition, bacterial plasmids are ideal for activating innate immunity as TLR-9 expressed on many phagocytic cells can recognize unmethylated bacterial DNA (see section "Adjuvants"). The main disadvantage of nucleic acid immunization is the poor immunogenicity in man. Therefore, they often require, like subunit vaccines, adjuvants or delivery systems to boost the immune response against the DNA-encoded antigen(s). Nevertheless, DNA has proven to be very effective when used in combination with protein antigens in heterologous DNA-prime/protein-boost strategies. The long-term safety of nucleic acid vaccines remains to be established. The main pros and cons of nucleic acid vaccines are listed in Table 14.5. Examples of DNA vaccines that have been tested in clinical trials comprise plasmids encoding HIV-1 antigens and malaria antigens.

mRNA Vaccines

In recent years, mRNA vaccines have gained increasing attention mainly because of their excellent safety profile, transient, non-integrative protein expression and enhanced immunogenicity as compared to plasmid DNA vaccines. mRNA vaccination is typically applied in oncology for the expression of mixtures of tumor antigens, but can also be applied for personalized vaccines. Initially, mRNA-based vaccines coped with stability problems and poor expression levels. To enhance immunogenicity and prolong protein expression, mRNAs were either chemically modified (both backbone and nucleoside modifications), sequence optimized, or formulated in

Advantages	Disadvantages
Low intrinsic immunogenicity	Effects of long-term expression unknown
Induction of long-term immune responses	Formation of antinucleic acid antibodies possible
Induction of both humoral and cellular immune responses	Possible integration of the vaccine DNA into the host genome
Possibility of constructing multiple epitope plasmids	Concept restricted to peptide and protein antigens
Heat stability	Poor delivery
Ease of large-scale production	Poorly immunogenic in man

Table 14.5 ■ Advantages and disadvantages of nucleic acid vaccines

nanocarriers (e.g., protamine nanoparticles). These modifications resulted in slower degradation and enhanced immune activation primarily through TLR7 signaling. Optimized mRNA vaccines have been shown to elicit strong and balanced Th1/Th2 immune responses in animal models. This technology is currently being tested in clinical trials, e.g., for the treatment of prostate cancer (Kubler et al. 2015) and non-small cell lung carcinoma (Sebastian et al. 2014), and has demonstrated antigen-specific immune responses in most patients.

One drawback of mRNA-based vaccines is their transient nature, often leading to short antigen expression times, unfavorable for proper immune activation. This can be circumvented by making use of self-amplifying RNAs based on the alphavirus replication machinery. Four alphavirus genes responsible for RNA replication are co-expressed with the gene of interest encoding the desired antigen (Fig. 14.7). Transfection of this single RNA construct into cells leads to prolonged and 10–50-fold enhanced antigen expression.

Delivery of Nucleic Acid Vaccines
Since nucleic acids do not easily enter cells but require intracellular delivery in their intact form for their activity, therapeutic application of these biomacromolecules requires sophisticated delivery methods or systems. A detailed description of nucleic acid delivery systems can be found in Chap. 16 on gene therapy.

For vaccination purposes, naked nucleic acids (i.e., without a delivery system) can be administered to animals and humans via intramuscular injection. The favorable properties of muscle cells for DNA expression are probably due to their relatively low turnover rate, which prevents that plasmid DNA is rapidly dispersed in dividing cells. After intracellular uptake of the DNA, the encoded protein is expressed on the sur-

face of host cells. After a single injection, the expression can last for more than a year. However, the use of naked DNA for vaccination requires high doses, most likely because of its poor delivery, and has so far shown poor immunogenicity in human trials.

Physical methods of DNA delivery can be used as well. These include ballistic approaches using a gene gun to inject DNA-coated gold nanoparticles into the epidermis, jet-injectors, electroporation and DNA tattooing (Samuels et al. 2017).

Delivery of nucleic acids with lipidic or polymeric nanocarriers can increase both the cellular uptake and immune activation. Nanocarriers protect the nucleic acids from premature degradation and enhance their cellular uptake by professional APCs. Besides synthetic nanocarriers, viruses can be used as vectors as well. A distinction can be made between replicating viruses and those that are replication incompetent. Examples of the latter are fowlpox and canarypox viruses that can infect mammalian cells, but are unable to replicate. Canarypox virus expressing HIV-1 rgp120 and rgp160 has been clinically tested as part of a heterologous prime/boost prophylactic HIV vaccine (O'Connell et al. 2016). Besides viruses, bacteria that replicate inside cells can also be used to deliver plasmid DNA into host cells for the expression of pathogen-derived antigens. Attenuated strains of *Shigella flexneri* and *Listeria monocytogenes* have been used for this purpose.

■ Therapeutic Vaccines
Most classical vaccine applications are prophylactic: they prevent an infectious disease from developing. Besides prophylactic applications, vaccines may be used to treat already established diseases, such as infectious diseases, cancer, and inflammatory disorders. Although the development of therapeutic vaccines is still in its infancy, especially in the field of cancer vaccines the insights and developments are rapidly progressing and some examples will be highlighted here.

Cancer Vaccines
Cancer is a collection of diseases characterized by uncontrolled cell division with the potential to invade and spread to other parts of the body. These characteristics are caused by gene mutations that are inherited or were accumulated during life by environmental factors. Such mutations may also lead to subtle changes in the antigenic repertoire of tumor cells as compared to healthy cells. This provides a basis for the development of therapeutic cancer vaccines aimed at inducing specific cellular immune responses and to a lesser extent humoral immune responses to pre-established cancer (Melief et al. 2015; van der Burg et al. 2016). A distinction can be made between so-called tumor-
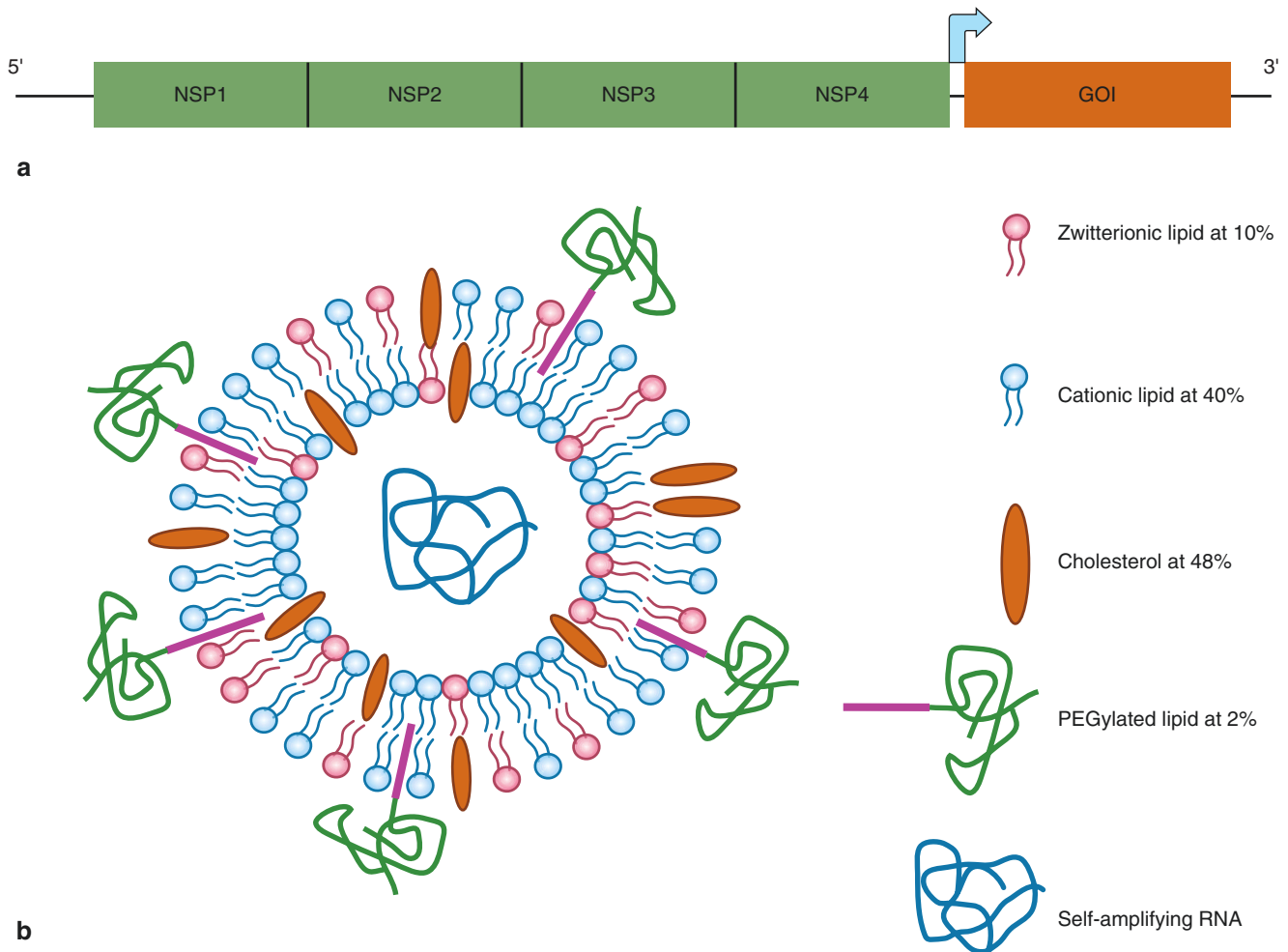
Figure 14.7 ■ Schematic illustration of an exemplary RNA vaccine. (**a**) Schematic illustration of an RNA construct encoding alphavirus-derived self-amplifying RNA. The RNA contains a 5′ cap, nonstructural genes for RNA replication (NSP1–4), a 26S subgenomic promoter (blue arrow), the gene of interest (GOI), and a 3′ polyadenylated tail. (**b**) Schematic illustration of a lipid nanoparticle encapsulating self-amplifying RNA, with the molar percentages of lipid components as indicated. Adapted from Geall et al. (2012)

associated antigens that are present in normal tissues, but over expressed in tumors, and neoantigens, which are newly formed tumor-specific antigens caused by somatic DNA mutations.

Tumor-Associated Antigen Vaccines
Initially, clinical trials with cancer vaccines focused on the use of a single tumor-associated antigen (e.g., melanoma-associated antigen-1, prostate-specific antigen, mucin-1, carcinoembryonic antigen), mixtures of ill-defined antigens from whole tumor cell lysates, or whole tumor cells. The latter can be autologous tumor cells directly isolated from the patients or allogeneic tumor cells that have been genetically modified to express cytokines (e.g., GM-CSF) or other immune-stimulating molecules. An advantage of using whole tumor cells is the presence of a wide array of tumor-specific antigens that could potentially lead to tumor-

specific immune responses. A disadvantage is that ill-defined tumor cell lysates will mostly express self-antigens. Breaking immunological tolerance against these self-antigens can result in transient or persistent autoimmune reactions.

Neoantigen Vaccines
Neoantigens are preferred for use in cancer vaccines, as they are foreign protein sequences that are absent in healthy tissue. However, since most neoantigens are unique to an individual's tumor, neoantigen vaccination requires a personalized approach, in which the vaccine composition is adjusted to the patient's needs. This is a labor intensive and costly procedure which must be performed fast because the patient is waiting for treatment.

Various neoantigen vaccination platforms have entered the clinic for the treatment of various cancers. Synthetic long peptide (SLP) vaccines consist of sets of

peptides containing both Th and CTL neoepitopes that need to be processed by professional APCs and cross presented on MHC class I in order to elicit antigen-specific cellular responses. An advantage of SLPs over synthetic peptide epitopes that can directly bind MHC class I molecules is that the need for antigen processing prevents T-cell anergy. Since the length of peptides that can be synthesized has its technical limitations, multiple SLPs need to be manufactured separately and combined to cover the breadth of neoantigens identified per individual. SLP vaccines have been successfully applied as therapeutic vaccines to treat cervical cancer as well as melanoma (Ott et al. 2017). Neoantigens can also be delivered as nucleic acids (both DNA and mRNA). An advantage of this approach is the intrinsic adjuvant properties of bacterially-derived plasmid DNA and mRNA and the ease at which multiple epitopes can be combined in a single construct. In addition, endogenous expression of antigen leads to efficient MHC class I presentation and subsequent CD8⁺ T-cell induction.

Both SLP- and nucleic acid-based approaches can also be used for application in an ex vivo setting, in which patient-derived DCs are loaded with the antigen source and stimulated with cytokines before being administered to the patient (see also Chap. 17). Overall, the results with neoantigen vaccination look promising with reported partial and complete cancer regressions in several trials.

Other Therapeutic Vaccine Applications

Besides prevention of infectious diseases or treatment of cancer, vaccines are also being developed for other therapeutic applications. These include treatment of Alzheimer's disease, induction of tolerance against food components and prevention of drug abuse. Most of these vaccines are still in an experimental phase. A few of these developments will be highlighted below.

Tolerogenic Vaccines to Treat Allergy or Autoimmune Diseases

Vaccines can be designed to induce immunological tolerance via the generation of regulatory T-cells (Tregs) with the aim to durably suppress undesired immune responses. For example, patients with autoimmune diseases in which the immune system attacks self-antigens and causes irreversible damage of tissues and cells would benefit from a vaccine that could specifically induce tolerance to the self-antigens. For multiple sclerosis, the self-antigen is known and several vaccination approaches have been followed to induce tolerance. These range from injection of T-cell epitopes derived from self-antigens to vaccination with tolerogenic nanoparticles containing self-antigens and immunosuppressive drugs (Hunter et al. 2014; Northrup et al. 2016). Similarly, the administration of low doses of antigens, also called allergy-specific immunotherapy, to desensitize against food (e.g., shrimp, peanut, cow's milk) or other (e.g., birch pollen, house dust mite) allergies are applied (Shamji and Durham 2017; Berings et al. 2017). Although the mechanism of desensitization remains largely unknown, Tregs probably play an important role.

Vaccines Against Alzheimer's Disease

Alzheimer's disease is a neurodegenerative disease characterized by amyloid plaque formation in the brain caused by aggregated amyloid-β, cleavage products of amyloid precursor protein as well as other proteins. Vaccines that induce antibodies against the aggregated form of amyloid-β or against the microtubule-associated protein tau have been tested in clinical trials. Initial trials suffered from serious side effects due to T-cell activation, but later trials circumvented this problem and showed good safety profiles (Novak et al. 2017). From these studies we have learned that antibody responses play a role in slowing down disease progression. However, the immunology of Alzheimer's disease is complex and far from understood (Dansokho et al. 2016). Also, it may be very difficult to reverse the damage caused by plaque formation by vaccination and therefore early diagnosis and treatment of patients with Alzheimer's disease is important.

ROUTE OF ADMINISTRATION

■ Introduction

The immunological response to a vaccine is dependent on the route of administration. Most current vaccines are administered intramuscularly or subcutaneously. Parenteral immunization (here defined as administration via those routes where a conventional hypodermic needle is used) usually induces systemic immunity but has disadvantages compared to other routes, e.g., needle phobia, infections caused by needlestick injuries and needle re-use, required vaccine sterility and injection skills. Moreover, parenterally administered vaccines generally do not result in effective immune responses at mucosal surfaces. As mucosal surfaces are a common port of entry for many pathogens, induction of a mucosal secretory IgA response may prevent the attachment and entry of pathogens into the host. For example, antibodies against cholera need to be in the gut lumen to inhibit adherence to and colonization of the intestinal wall. Therefore, mucosal (e.g., oral, intranasal, or intravaginal) immunization may be preferred, because it may induce both mucosal and systemic immunity. For instance, orally administered live attenuated *Salmonella typhi* vaccine not only invades the mucosal lining of the gut but also infects cells of the phagocytic system throughout the body, thereby stimulating the production of both secretory and systemic antibodies. Additional advantages of

mucosal immunization are the ease of administration and the avoidance of systemic side effects (Czerkinsky and Holmgren 2012; Holmgren and Czerkinsky 2005).

■ The Oral Route of Administration

From a receiver perspective, oral delivery of vaccines would be preferable in many cases, because it is vaccinee friendly. Up to now, however, only a limited number of oral vaccines (e.g., oral polio, cholera, typhoid fever, and rotavirus vaccines) have made it to the market. Most of these vaccines are based on attenuated versions of pathogens for which the route of administration is the same as the natural route of infection. The gut is relatively immune tolerant to prevent immune responses against food antigens. Therefore, a relatively high dose of antigen is required to induce significant responses. A replicating vaccine provides this more easily than an inactivated vaccine. In addition, oral bioavailability is usually very low because of (1) degradation of protein antigens in the gastrointestinal (GI) tract and (2) poor permeability of the wall of the GI tract in case of a passive transport process.

Still, for the category of oral vaccines, the above-mentioned hurdles of degradation and permeation are not necessarily prohibitive. For oral immunization, only a (small) fraction of the antigen has to reach its target site to elicit an immune response. The target cells are lymphocytes and antigen-presenting accessory cells located in Peyer's patches (Fig. 14.8). The B-lymphocyte population includes cells that produce secretory IgA antibodies.

These Peyer's patches are macroscopically identifiable follicular structures located in the wall of the gastrointestinal tract. Peyer's patches are overlaid with microfold (M) cells that separate the luminal contents from the lymphocytes. These M cells have little lysosomal degradation capacity, are specialized in the uptake of particulate matter, and allow for antigen sampling and delivery to underlying APCs. Moreover, the density of mucus-producing goblet cells is lower in Peyer's patches than in surrounding parts of the GI tract. This reduces mucus production and facilitates access to the M cell surface for luminal contents (Delves and Roitt 2011), which is of particular importance for the uptake of nano- and microparticle based vaccines. Consequently, attempts to improve antigen delivery via the Peyer's patches and to enhance the immune response are made by using microspheres, liposomes, or modified live vectors, such as attenuated bacteria and viruses (Vela Ramirez et al. 2017). The latter have the additional advantage of replication induced dose increase.

■ Other Routes of Administration

Apart from the oral route, the nose, lungs, rectum, oral cavity, and skin have been selected as potential sites of non-invasive vaccine administration. Most vaccines administered via these routes are still under develop-
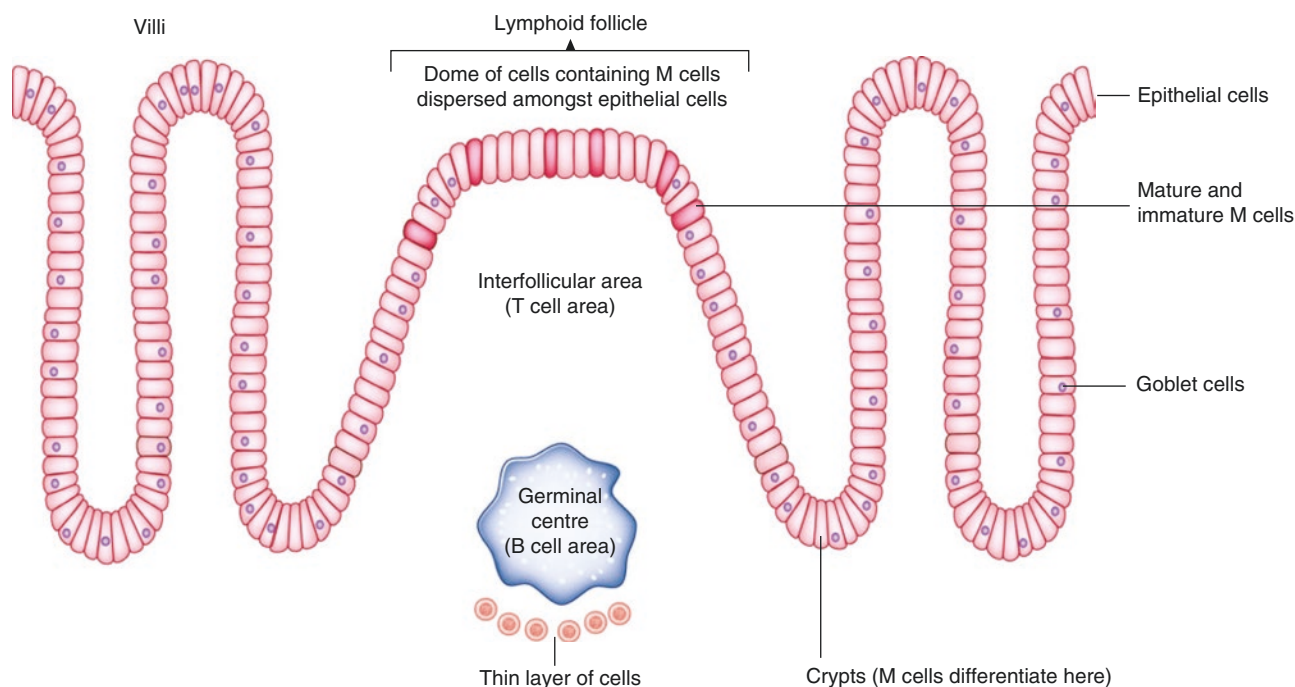

Figure 14.8 ■ Schematic diagram of the structure of intestinal Peyer's patches. M cells within the follicle-associated epithelium are enlarged for emphasis (from O'Hagan 1990)

ment. Today, only a nasal influenza vaccine is licensed (FluMist, branded as Fluenz in Europe).

Besides mucosal vaccines, a number of intradermal vaccine delivery systems have been developed. These include needle-free jet injection of vaccines in liquid form and intradermal delivery with microneedles (Kersten and Hirschberg 2004; van der Maaden et al. 2012; Hogan et al. 2015). Up to now, these products have not yet been registered although an influenza vaccine given intramuscularly with a fluid jet injector is licensed in the US. Intradermal jet injectors are in clinical development, e.g., for inactivated polio vaccine. The classical liquid jet injectors deliver small volumes (microliter range) of liquid vaccine formulation with a high velocity. Depending on fluid velocity and nozzle design, the vaccine is deposited intradermally or dispersed deeper, i.e., subcutaneously of intramuscularly. Current versions use prefilled disposable delivery units for single use to avoid contamination.

Another attractive, potentially pain free approach for intradermal vaccine delivery is the use of microneedles or microneedle arrays with small individual needles in the 100–1000 μm range. There are multiple microneedle types and formats, such as solid microneedle arrays on which the vaccine components are coated, hollow microneedles through which a liquid vaccine formulation can be delivered via a micropump or syringe, and dissolvable microneedles containing the antigen/adjuvant embedded in, e.g., a sugar or polymer matrix, which dissolves rapidly after application (Mitragotri 2005; Kis et al. 2012; van der Maaden et al. 2012). Examples are shown in Fig. 14.9.

PHARMACEUTICAL ASPECTS

■ Production

Except for synthetic peptides, the antigenic components of vaccines are derived from microorganisms or animal cells. For optimal expression of the required vaccine component(s), these microorganisms or animal cells can be genetically modified. Animal cells are used for the cultivation of viruses and for the production of some subunit vaccine components and have the advantage that the vaccine components are released into the culture medium. However, some viruses cause cell lysis and consequently the culture medium will contain high concentrations of host cell proteins and host cell DNA, requiring extensive purification steps.

Three stages can be discerned in the manufacture of cell-derived vaccines: (1) cultivation or upstream processing, (2) purification or downstream processing, and (3) formulation. For the first two stages, the reader is referred to Chap. 4 and formulation of biopharmaceuticals is addressed in Chap. 5. The following section deals with formulation aspects specifically related to vaccines.
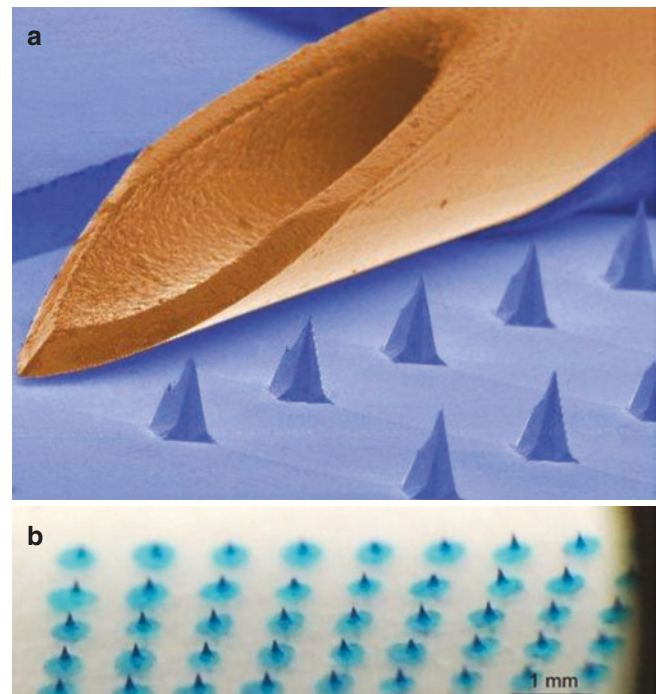

Figure 14.9 ■ (**a**) Hollow silicon microneedles, 300 μm in length, fabricated using a combination of wet and dry etch micromachining technologies (blue) and a 26-gauge syringe needle (brown) for comparison (Courtesy: Joe O'Brien & Conor O'Mahony, Tyndall National Institute). (**b**) Array of dissolvable microneedles, 280 μm in length at a density of 144 needles per cm², composed of sugars and polymers, fabricated in PDMS molds of master silicon microneedle arrays using a proprietary method (UK Patent Application Number 1107642.9) (Courtesy: Anne Moore, Anto Vrdoljak, School of Pharmacy, University College Cork)

■ Formulation

Adjuvants: Immune Potentiators and Delivery Systems

The formulation of the vaccine is one of the major determinants that influence the type of immune response that is elicited, as it determines the type of co-stimulatory molecules and cytokines that are expressed by APCs. Through their various PRRs, APCs are more or less capable of "sensing" the type of vaccine that is encountered. This determines the set of co-stimulatory signals and proinflammatory cytokines that APCs will generate when presenting the antigen to T-cells in the peripheral lymphoid organs (Pulendran and Ahmed 2011). For instance, pathogens or vaccines containing lipoproteins or peptidoglycans will trigger DCs via TLR-2, which predominantly generates a Th2 response, whereas stimulation of DCs through TLR-3, or TLR-9 is known to yield robust Th1 and CTL responses. Therefore, vaccines should be formulated in such a way that the appropriate T-cell response will be triggered. This can be done by presenting the antigen in its native format, as is the case for the live-attenuated vaccines, or by formulating the native antigen with adjuvants that stimulate the desired response.

Besides immune stimulatory molecules, a vehicle to deliver antigen to antigen presenting cells and B-cells may be crucial, especially for highly purified subunit antigens. Immune stimulatory molecules and delivery systems are called adjuvants. Adjuvants are defined as any material that can increase or modulate the immune response against an antigen. Adjuvants can stimulate the immune system by several, not mutually exclusive mechanisms (Guy 2007): (1) a depot effect leading to slow antigen release and prolonged antigen presentation, (2) attraction and stimulation of APCs by some local tissue damage and binding to PRRs present on or in APCs, and (3) delivery of the antigen to regional lymph nodes by improved antigen uptake, transport, and presentation by APCs.

Colloidal aluminum salts (hydroxide, phosphate) are widely used adjuvants in many classical vaccine formulations. A few other adjuvants, e.g., monophosphoryl lipid A in HPV vaccine and oil-in-water emulsions in influenza vaccines, have been introduced in marketed vaccines. Moreover, numerous adjuvants are in several stages of (pre)clinical testing or are used in veterinary vaccines. Table 14.6 shows some examples of well-known adjuvants.

Combination Vaccines

Since oral immunization is not possible for most available vaccines (see the section "Route of Administration" above), the strategy to mix individual vaccines in order to limit the number of injections has been common practice since many decades. Currently, vaccines are available containing up to six nonrelated antigens: diphtheria-tetanus-pertussis-hepatitis B-polio-Haemophilus *influenzae* type b vaccine. Another example is MMR vaccine, alone or in combination with varicella vaccine. Sometimes a vaccine contains antigens from several subtypes of a particular pathogen. Pneumococcal conjugate vaccine 13 (PCV13) is an example. This vaccine contains polysaccharides from 13 pneumococcal strains, conjugated to a carrier protein to provide T-helper cell recognition and, as a result, induce immunological memory.

Combining vaccine components sometimes results in pharmaceutical as well as immunological problems. For instance, formaldehyde-containing components may chemically react with other components; an unstable antigen may need freeze drying, whereas other antigens should not be frozen. Components that are not compatible can be mixed prior to injection, if there is no short-term incompatibility. To this end, dual-chamber cartridges (cf. Chap. 5) have been developed.

From an immunological point of view, the immunization schedules of the individual components of combination vaccines should match. Pertussis vaccine, for instance, should be given as early in life as possi-

Immune potentiators	Examples	Characteristics
Bacterial origin		
Triacyl lipopeptides	Pam3Cys; Pam3CSK4	TLR1–TLR2 agonists
Diacyl lipopeptides	Pam2Cys; MALP2	TLR2–TLR6 agonists
LPS analogs	MPL; RC-529	Endotoxins; TLR4 agonists
Cell wall components	Peptidoglycan; muramyl peptides	TLR2–TLR4 agonists
Flagellin		TLR5 agonist
CpG DNA		TLR9 agonist
Toxins	Cholera toxin B subunit; heat labile enterotoxin subunit B	
Viral origin		
Double stranded RNA	Poly(I:C); poly(rA:rU)	TLR3 agonists
Guanoside analogs	Imiquimod; resiquimod	TLR7–TLR8 agonists
Other origin		
Plant-derived	QuilA; QS21	Triterpene glycosides; crucial components of ISCOMs
Mineral	Aluminum hydroxide; aluminum phosphate	Colloidal suspensions; antigen adsorption is crucial
Synthetic lipids	Avridine; DDA	Used as liposome components
Delivery systems	*Examples*	*Characteristics*
Oil-in-water emulsions	AF03; MF59	
Water-in-oil emulsions	Montanide ISA 51; Montanide ISA 720	
Particulate carriers	Liposomes; virosomes; ISCOMs; polymeric nano- and microparticles; bacterial ghosts	Antigen association with carrier is cruical
Combination adjuvants	*Examples*	*Characteristics*
Miscellaneous	AS01; AS02; CAF01; Montanide ISA 51 plus GM-CSF	

[a]Adapted from Amorij et al. (2012)

Table 14.6 ■ Examples of adjuvants used in vaccine formulations[a]

ble, since whooping cough is most dangerous in very young children, whereas hepatitis B vaccine can be given later in life because it is mainly a sexually transmitted disease. Even when this condition of matching immunization schedules is met and the components are pharmaceutically compatible, the success of a combination vaccine is not warranted. Vaccine components in combination vaccines may exhibit a different behavior in vivo compared to separate administration of the components. For instance, enhancement (Paradiso et al. 1993) as well as suppression (Mallet et al. 2004) of humoral immune responses has been reported.

■ Characterization

Modern vaccines have to meet similar standards as other biotechnological pharmaceuticals and can be characterized with a combination of appropriate biochemical, physicochemical, and immunochemical techniques (cf. Chaps. 3 and 5). The use of state-of-the art analytical techniques for the design and release of new vaccines is gaining importance. Currently, animal experiments are needed for quality control of many vaccines but in vitro analytical techniques may eventually (partly) substitute tests in vivo. During the development of the production process of a vaccine component, a combination of suitable assays can be defined. These assays can subsequently be applied during its routine production.

Column chromatographic (HPLC) and electrophoretic techniques, such as gel electrophoresis and capillary electrophoresis, provide information about the purity, molecular weight, and other physicochemical properties of antigens. Physicochemical assays comprise mass spectrometry and spectroscopy, including circular dichroism and fluorescence spectroscopy. Information is obtained mainly about the molecular weight and the conformation of the antigen(s). Immunochemical assays, such as enzyme-linked immunoassays, are powerful methods for the quantification of the antigen(s). By using monoclonal antibodies (preferably with the same specificity as those of protective human antibodies) information can be obtained about the conformation and accessibility of the epitope to which the antibodies are directed. Moreover, the use of biosensors makes it possible to measure antigen-antibody interactions momentarily, allowing accurate determination of binding kinetics and affinity constants. Furthermore, since practically all vaccines are particulate in nature, it is sensible to use state-of-the-art particle sizing and counting methods to characterize them (Slütter and Jiskoot 2016).

■ Storage

Depending on their specific characteristics, vaccines are stored as solution or as a freeze-dried formulation, usually at 2–8 °C. Their shelf life depends on the composition and physicochemical characteristics of the vaccine formulation and on the storage conditions and typically is in the order of several years. The quality of the primary container can influence the long-term stability of vaccines, e.g., through adsorption or pH changes resulting from contact with the vial wall or vial stopper. The use of pH indicators or temperature- or time-sensitive labels ("vial vaccine monitors," which change color when too long exposed to too high temperatures) can avoid unintentional administration of an inappropriately stored vaccine.

CONCLUDING REMARKS

Despite the tremendous success of the classical vaccines, there are still many infectious diseases and other diseases (e.g., cancer) against which no effective vaccine exists. Although modern vaccines—like other biopharmaceuticals—are expensive, calculations may indicate cost-effectiveness for vaccination against many of these diseases. In addition, the growing resistance to the existing arsenal of antibiotics increases the need to develop vaccines against common bacterial infections. It is expected that novel vaccines against several of these diseases will become available, and in these cases, the preferred type of vaccine will be chosen from one of the different options described in this chapter.

SELF-ASSESSMENT QUESTIONS

■ Questions

1. Imagine three vaccine types against the same viral disease: (1) formaldehyde inactivated virus, (2) genetically attenuated live virus and (3) highly purified viral protein.
   (a) One vaccine is supplemented with an adjuvant. What is an adjuvant?
   (b) Which of the three vaccines should contain added adjuvant and why?
   (c) Vaccines 1 and 2 have almost the same antigen composition. Despite this, one vaccine can be given in a considerably lower dose than the other one to induce the same level of protection. Which one and why?
   (d) Which vaccine is able to induce cellular cytotoxic T-cell responses and why?
2. How do antibodies prevent infection or disease?
3. How does the immune system prevent unwanted T-cell responses against self-antigens and how does this affect vaccine design?
4. What is the definition of a subunit vaccine? Give three different types of subunit vaccines.
5. Mention at least three advantages and three disadvantages of nucleic acid vaccines. Give one advan-

tage and one disadvantage of RNA vaccines over DNA vaccines.

6. Mention at least three advantages of mucosal vaccination. What are M cells and why are they important in mucosal vaccination?

7. Mention two or more examples of currently available combination vaccines. Which pharmaceutical and immunological conditions have to be fulfilled when formulating combination vaccines?

■ Answers

1.

(a) An adjuvant is a vaccine component improving qualitatively and/or quantitatively the immune response against an antigen. Adjuvants act on the innate immune system.

(b) Vaccine 3, because it only contains pure antigen and lacks an innate immune stimulus. Vaccines 1 and 2 consist of complete viruses which in general contain innate immune potentiators, such as double stranded RNA.

(c) Vaccine 2 is a live vaccine. Therefore, it can replicate to some extent after administration, increasing the effective dose and extending the contact time with the immune system.

(d) Vaccine 2, because it infects cells. Infected cells produce progeny virus. This endogenous antigen source is partially processed and presented in MHC class 1 molecules to Th-cells. This results in induction of CD8 T-cells.

2. Antibodies are able to neutralize pathogens by at least four mechanisms:
   (a) Fc-mediated phagocytosis
   (b) Complement activation resulting in cytolytic activity
   (c) Complement-mediated phagocytosis
   (d) Competitive binding on sites that are crucial for the biological activity of the antigen

3. Besides antigen presentation through MHCI or MHCII molecules, T-cells require a second signal from an APC before they will proliferate. This second signal supplied by the APC is referred to as co-stimulation and only occurs when the APC has sensed danger, by detecting PAMPs. Therefore, an effective vaccine needs to contain both an antigen and a PAMP (often in the form of an adjuvant).

4. Subunit vaccines are vaccines that contain one or more individual components of a pathogen, e.g., proteins, oligosaccharides or peptide epitopes. These can be either isolated from the pathogen (in case of oligosaccharides, toxins or other protein antigens), recombinantly produced (in case of protein antigens) or synthesized (in case of peptide epitopes).

5. The advantages and disadvantages of nucleic acid vaccines are listed in Table 14.6. An advantage of RNA is that there is no risk of incorporation into host DNA. A disadvantage of RNA is that it is less stable than DNA.

6. Advantages of mucosal vaccination over vaccination by injection are that it:
   (a) avoids infections caused by needlestick injuries and needle re-use
   (b) is easier to perform and more vaccinee friendly
   (c) can induce mucosal immunity
   M cells are cells present in mucosal surfaces (such as the nasal cavity and the Peyer's patches in the gastrointestinal tract). M cells have little lysosomal degradation capacity, are specialized in the uptake of particulate matter, such as nano- and microparticulate vaccines. They can sample particulate antigens and deliver them to underlying APCs. The density of mucus-producing goblet cells is low in Peyer's patches and M cells do not produce mucus, which facilitates the access of (particulate) antigens to the M cell surface.

7. Examples of combination vaccines include diphtheria-tetanus-pertussis(−polio) vaccines and measles-mumps-rubella(−varicella) vaccines. Prerequisites for combining vaccine components are:
   (a) Pharmaceutical compatibility of vaccine components and additives
   (b) Compatibility of immunization schedules
   (c) No interference between immune responses to individual components

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