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PRINCIPLES OF CELLULAR DRUG DELIVERY

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Augmentation of Cell-Mediated Immunity to Virus

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1. Immune Responses to Viral Infection

Although most infections due to self-replicating pathogens such as bacteria and fungi can be treated by drug therapy and cleared by subsequent host antibody responses, viral infection may require additional defense mechanisms known as cell-mediated immunity. Unlike bacteria and fungi, virus replication requires host-cell machinery, including mechanisms of DNA, RNA, and protein synthesis. Therefore, after initial viral infection of host cells and tissues, viral RNA or DNA may persist in infected but viable cells. At an opportune time, viral replication is initiated, and the infected host cells are lysed and viral progeny released. These processes are observed clinically as an active, or recurring, viral infection and pathogenesis in the host. Although antibody, or humoral, responses elicited during the first exposure (primary infection) may help clear the reactivated virus, such mechanisms may develop well after severe consequences to the host have occurred. Therefore, it is important to control viral reactivation by selectively eliminating virus-infected cells, which is a specialized function of cell-mediated immunity. In order to elicit a cell-mediated response, select fragments of viral antigens must be presented on the surface of infected cells, along with major histocompatibility complex (MHC) molecules. MHC molecules are polymorphic glycoproteins that bind antigen and mediate migration of the antigen to the cell surface to be presented to immune cells. The induction of cell-mediated responses, which can directly or indirectly kill virus-infected cells, is critical in clearing viral infection from the body.

The unpredictable reoccurrence of herpes simplex virus (HSV) is an example in which the presence of humoral responses is not sufficient to clear infection, highlighting the role of cell-mediated immunity. The two major strains of HSV are HSV-1, which primarily infects the lips and manifests in cold sores, and HSV-2, which produces genital lesions. Primary HSV-1 infection is followed by a stage of latency, or a dormant state, where virus sequences can be found in neurons of the trigeminal ganglion (1). HSV-1 infection starts in epithelial cells, causing a cold sore, and spreads to sensory neurons, whose axons innervate the lips. The initial infection is cleared by immune responses but HSV-1 then becomes latent in these neurons. In this latent state, HSV-1 does not replicate or synthesize protein and, therefore, exists in infected cells with low antigen density that is insufficient to trigger a cell-mediated response. In

addition, HSV-1 and HSV-2 have been shown to avoid recognition by cell-mediated responses by expressing a viral protein called ICP47, which inhibits viral antigen processing and presentation through class I MHC (MHC-I) molecules on the surface of infected cells (2). Although HSV-1 and HSV-2-infected individuals have circulating antibodies against HSV-1, they are unprotected from recurrences (3). All of these data point to the requirement of functional cell-mediated immunity to control HSV viral replication and reactivation.

The immune system consists of distinct cell types with specialized roles at different stages of the immune response (4). The differentiated immune cells are derived from hematopoietic stem cells, which are localized primarily in bone marrow. Immune system cells are found in the spleen, lymph nodes, tonsils, bone marrow, blood, thymus, and Peyer's patches in the intestine. White blood cells, including macrophages, dendritic cells (DCs), Langerhans cells, natural killer (NK) cells, mast cells, and basophils may interact with lymphocytes in the presentation of an antigen and thereby mediate the immune response. Two classes of lymphocytes, central to immunity against pathogenic challenge, are T lymphocytes (T cells), which mediate cell-mediated responses and B lymphocytes (B cells), which are important primarily in mediating humoral responses. Some of the key functions of T lymphocytes are regulation of the development of certain types of immune responses, such as autoimmune response, graft-v-host reactions, facilitation of antibody production, enhancement of the microbicidal activity of macrophages, and lysis of virus-infected cells and certain cancer cells. The B lymphocytes are precursors of antibody-secreting cells and may be involved in antigen presentation to T lymphocytes. When a naive B cell binds to an antigen, the cell rapidly divides and differentiates into mature B cells, called plasma cells. Plasma cells secrete immunoglobulins, which are antibody molecules that exhibit antitoxin activity and thereby neutralize pathogens. Immunoglobulins are found at high concentrations in blood or plasma. When viral particle levels subside in the host, the plasma cells stop secreting antibodies, but a small fraction of cells specific for that particular antigen, called memory B-cells, continue to exist. Additional details on the biology of antibodies and immunoglobulins and their therapeutic actions have been recently reviewed (5).

Although the humoral response, mediated by antibody-secreting B cells, is an important component to combating viral infection, its effectiveness may be limited to clearance of extracellular viruses and to some extent preventing viral entry by processes of neutralization, complement activation, and opsonization (6). Intracellular virus, either as residual or latent infection in host cells, must be controlled or eliminated selectively in order to completely recover from viral infection. Our immune system has developed antigen-specific cell-mediated mechanisms to seek out and clear infected cells. One of the key responses is the ability of cytotoxic T lymphocytes (CTLs) to recognize and produce contact-dependent cell-mediated lysis of virus-infected host cells. Because CTLs exhibit exquisite recognition of specific antigens and safeguards against autoimmune reactions, their mechanisms of action and capacity for clonal expansion have been well-studied over the past three decades. Figure 1 displays a simplified schematic of the interrelationships between antigen-presenting cells (APCs), and T and B lymphocytes, leading to humoral and cell-mediated responses.

In this chapter, we will briefly review the biological processes of a cell-mediated response and discuss strategies aimed at promoting cell-mediated responses against viral infection. We will also discuss how vaccine design can be improved to elicit cell-mediated immune responses through selective tissue and cell-delivery strategies.

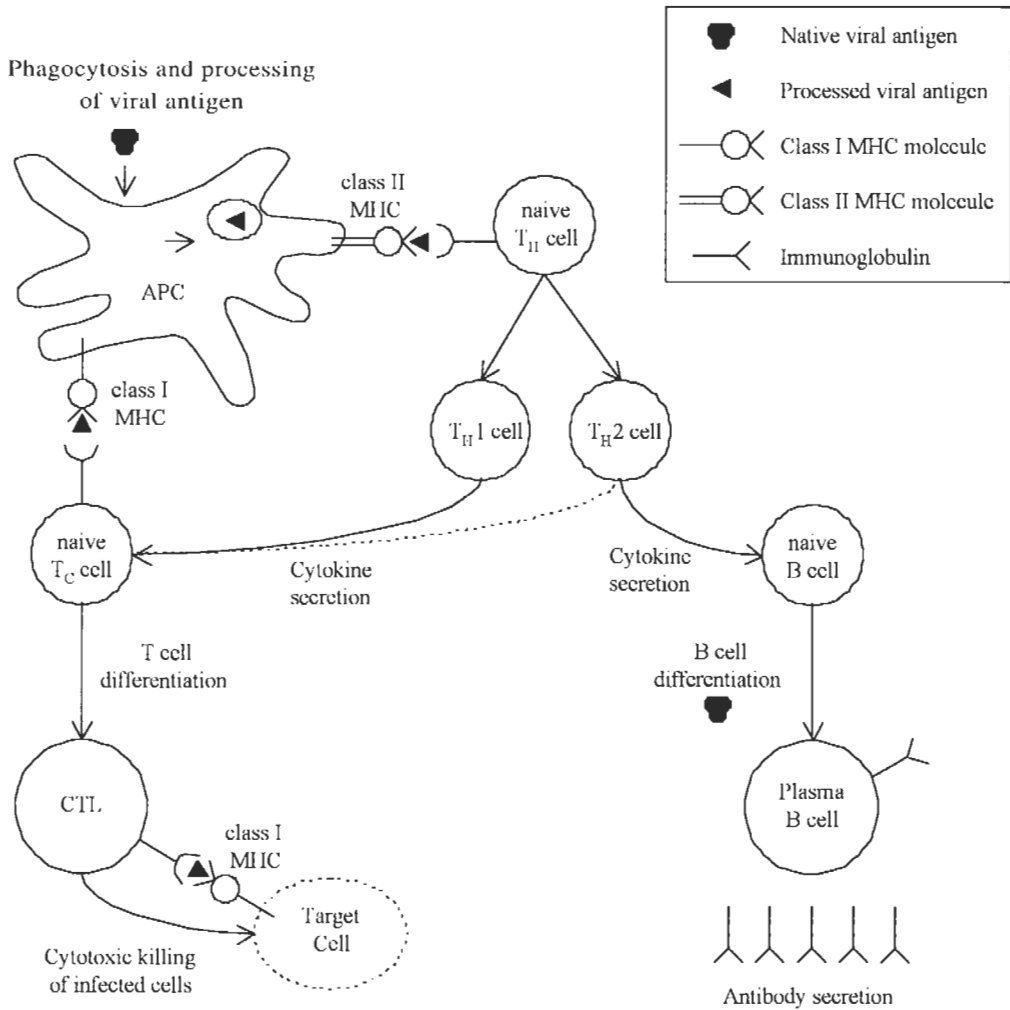


Fig. 1. Schematic representation of the relationships between humoral and cell-mediated responses to viral infection. Viral antigen is phagocytosed by APCs, and processed and presented with MHC-II molecules on the cell membrane. MHC-II molecules trigger differentiation of naive Th (or T_H) cells into two subpopulations, Th1 and Th2, which secrete a variety of cytokines. Th1 cells secrete cytokines that primarily induce the differentiation of naive T_C cells into cytotoxic T lymphocytes, which seek out and lyse virus-infected cells expressing MHC-I molecules. Cytokines secreted by Th2 primarily promote naive B cells to differentiate into plasma cells that secrete large amounts of antibody specific for the viral pathogen. Live-attenuated vaccines generate an immune response similar to natural infection, initiating both humoral and cell-mediated responses. Without the enhancement of antigen-delivery systems and adjuvants, inactivated and subunit antigens are not optimally processed and presented in APCs and, therefore, primarily generate an antibody response through stimulation of B cells.

Advances in recombinant vaccine technologies have produced virus-free vaccines that are safe, but in need of enhanced cell-mediated responses. We will discuss some delivery systems and adjuvants that have been developed to augment the capacity and magnitude of cell-mediated responses, thereby influencing vaccine safety and efficacy.

2. Role and Mechanisms of Cell-Mediated Immunity

The cellular response to viral infection can be roughly divided into a nonspecific component, called innate immunity, and a specific component, called adaptive immunity. The innate immune response relies on a rather nonspecific recognition of most pathogens and is quickly activated, but it is often not sufficient to eliminate an infection. The innate system provides some control of the infection while the cells of the adaptive immunity are being activated. Typically, there is a 5- to 6-d delay between infection and the initiation of the specific adaptive, or acquired, immune response. Mounting an effective immune response against infection often requires selective recognition of a pathogen. Pathogen-specific immunity is provided by adaptive cell-mediated responses including CTL cell-dependent clearance of virus-infected cells. These effector cells are also maintained as memory cells specific for the particular pathogen. Viruses often have an ability to evade certain aspects of the immune responses. Therefore, duration and extent of viral infection would depend on the ability of the host to overcome the dynamics of virus–host interactions.

Mechanisms of both innate and adaptive immune systems work in concert to provide optimal immune responses in clearing extracellular virus and virus-infected cells. Individuals lacking effective innate immunity are not able to contain the initial stages of viral infection and, therefore, the virus replicates uncontrollably. Individuals lacking adaptive immunity are able to limit initial viral replication through processes of innate immunity, but ultimately are not able to clear infection without a specific response to the pathogen. Therefore, both mechanisms are essential for clearing viral infection from the body. The ability of cell-mediated and humoral responses to eliminate viral infection is discussed further in Box 1 using influenza infection as an example.

2.1. Innate Immunity Contributes to Cell-Mediated Immunity

Instead of specific recognition of a pathogen, mechanisms of the innate immune response rely on recognition of common features of a wide spectrum of pathogens or components of the immune system bound to pathogens. Innate immunity, unlike adaptive immunity, is not increased by exposure to a pathogen. An important element of the innate immunity is phagocytosis, which involves internalization, degradation, and presentation of antigen fragments on the cell surface. Phagocytic cells of the immune system include macrophages, dendritic cells, and neutrophils. Macrophages, dendritic cells, and B cells can act as professional antigen-presenting cells (APCs), which bind antigenic peptides from the ingested pathogen to MHC molecules and present them on the cell membrane. MHC molecules consist of two major types: MHC-I molecules, expressed on nearly all nucleated cells, and class II MHC (MHC-II) molecules, expressed only by APCs. APCs are important in communicating with the adaptive immune system by eliciting or recruiting T helper (Th) cells and CTLs, and play a major role in both humoral and cell-mediated immune responses.

The major antiviral activities of the innate response are the induction of cytokines and the activation of NK cells. A human cell infected with virus will secrete interferon- α (IFN- α) and interferon- β (IFN- β) (7). The expression of IFN- α and IFN- β is triggered by double-stranded RNA, which is required for virus replication and not normally found in human cells. The antiviral response generated by IFN- α and IFN- β is mediated by binding to IFN- α/β receptors, which induces the expression of a ribonuclease that degrades viral RNA, and the expression of a protein kinase that inactivates viral

protein synthesis. IFN- α and IFN- β also activate NK cells, important for combating viral infection. Interleukins 2 (IL-2) and 12 (IL-12) are also important in antiviral activity: IL-2, secreted by Th cells, is important in recruitment of CTLs and IL-12 is important in activation of NK cells.

NK cells are another important component of innate immunity, and are particularly crucial in containing viral infections through cell-mediated lysis of infected cells. NK cells are nonspecific lymphocytes recruited to the site of infection by inflammatory cytokines. The primary role of NK cells is to control viral replication through cytotoxic activity and the secretion of cytokines until cells of the adaptive immunity have been activated. NK cells also secrete tumor necrosis factor (TNF), which can further enhance their cytotoxic effect. NK cells can also be activated by the humoral response causing apoptosis in virus-infected cells in a process known as antibody-dependent cell-mediated cytotoxicity (ADCC). Target-cell killing by ADCC involves the binding of Fc receptors expressed on NK cells to the Fc region of IgG antibody molecules bound to the surface of infected cells. Macrophages, monocytes, neutrophils, and eosinophils can also mediate ADCC by similar mechanisms. NK cells are an important component in combating viral infection, especially in the early phase of infection before CTLs are developed.

2.2. Antigen Specific Cell-Mediated Immunity

Once an infection is established, cell-mediated responses are crucial in clearing virus-infected cells from the body and eliminating the infection. Cell-mediated responses are generated by various subpopulations of T cells. When a naïve T cell encounters an antigen presented with MHC, it differentiates into memory and various effector T cells. Effector T cells are the most important component of cell-mediated immunity and express a T-cell receptor that recognizes specific antigen epitopes bound to MHC molecules. Antigen-specific T cells are selected and expanded based on the particular viral antigen. The two main subpopulations of T cells, T helper (Th), and T cytotoxic (Tc) cells, work together to produce cell-mediated responses. Th cells express the membrane glycoprotein CD4 and are therefore also known as CD4⁺ T cells. Th cells recognize antigen displayed with MHC-II molecules on the surface of APCs, becoming activated to cytokine-secreting effector cells. Cytokines play an important role in activating and expanding cell-mediated immunity by inducing phagocytosis and activating B and Tc cells. Th cells are further subdivided into Th1 and Th2 according to their cytokine secretion profile. Th1 cells secrete IL-2, IFN- γ , and TNF- β , and are important in the activation of many cell-mediated responses including hypersensitivity reactions and CTL stimulation. Th2 cells secrete IL-4, IL-5, IL-6, and IL-10, and function as activators of B cells. Both IFN- γ and IL-2 activate NK cells; their importance in controlling viral infection was discussed in a previous section.

The second class of T lymphocytes, Tc cells, express the glycoprotein CD8 and are often referred to as CD8⁺ T cells. Tc cells are activated by the recognition of MHC-I-presented antigens and by cytokines secreted by Th cells, and differentiate into CTL effector cells. CTLs secrete few cytokines; their primary function is to exhibit cytotoxic activity. Because nearly all nucleated cells express MHC-I-molecules, CTLs can recognize many types of virus-infected cells virtually anywhere in the body. The major function of CTLs in viral infection is to mediate contact-dependent cell-mediated lysis of infected cells, thereby eliminating the source of viral replication and reactivation.

CTL lysis is one of the most potent immune mechanisms for clearing a viral infection from the body (8,9).

Box 1. The Role of Cell-Mediated Immunity in Influenza Infection

A classic example of viral infection that can be completely cleared by the body's humoral and cell-mediated immune systems is influenza infection, which has been extensively reviewed in the literature (10,11). Influenza is a rapidly replicating virus; therefore, it is an effective stimulator of cell-mediated responses and is also efficiently cleared by CTLs and antibodies. Influenza viral particles are surrounded by an outer envelope, which is covered with two glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Both of these glycoproteins have unique functions that allow them to infect and replicate in host cells. HA binds to sialic acid groups on the plasma membrane of host cells facilitating cell entry. NA cleaves *N*-acetylneuraminic (sialic) acid from viral and host-cell glycoproteins to facilitate viral budding from the host cell. A distinctive feature of influenza virus is the antigenic variation observed between different subtypes. The influenza virus has the ability to genetically modify the genes encoding HA and NA glycoproteins expressed on its surface so that the immune responses to different subtypes are completely altered.

Clearance of the influenza virus from the body involves both the innate and adaptive arms of the immune system. Innate mechanisms include the initiation of fever, production of interferon- α (IFN- α) and IFN- β , macrophages, NK cells, and complement. Influenza virus titer does decline in the early phase of infection; however, there is persistence of the virus, indicating that innate immunity is not sufficient for recovery. The humoral response to influenza is generated against the HA glycoprotein, but this specificity is dependent on the individual's prior antigenic exposure. However, adults who have been previously exposed to a different subtype produce cross-reactive antibodies, as well as strain-specific antibodies. The humoral response is capable of neutralizing the virus before infection of host cells and mediating the lytic action of ADCC in cells that have already become infected. However, individuals lacking antibodies to HA are still able to recover from influenza infection, indicating that cell-mediated responses are also important. Memory T cells, both species-specific and cross-reactive, correlate with rapid clearing of the virus. Effector T cells, including CTLs, are critical in the removal of infected cells and in the recovery from infection.

3. Immune Responses Elicited by Viral Vaccines

After an infection subsides, most effector B and T lymphocytes die; however, memory cells continue circulating and form the basis for protection against the same pathogen in the future. Vaccines often mimic natural infection by establishing pathogen-specific immune memory in vaccinated individuals. When a successfully vaccinated individual is exposed to the live pathogen, their immune system is armed and ready, and more likely to mount a rapid response. Often conventional vaccines are empirically designed to produce sufficient immunity. Vaccine antigens could be derived from live-attenuated viruses, inactivated (or killed) viruses, or recombinant sub-

Table 1
Current Viral Vaccines

Live-attenuated	Inactivated or killed	Subcellular/subunit
Adenovirus	Hepatitis A	Hepatitis A
Influenza (cold-adapted)	Influenza	Hepatitis B
Measles/Mumps/Rubella	Japanese encephalitis	HIV-1 ^a
Polio	Polio	HSV-2 ^a
Smallpox	Rabies	Influenza
Varicella zoster		
West Nile ^a		
Yellow fever		

^aVaccines in clinical trials.

unit fractions to elicit protective immune responses. Vaccines approved for human use in the United States, and those in development, are listed in Table 1.

Many US Food and Drug Administration (FDA)-approved viral vaccines provide sufficient antibody responses to abort the primary infection; for example, the influenza vaccine composed of inactivated or killed antigen and the alum adjuvant. Even with the help of the only adjuvant approved for human use, alum-induced inactivated viral antigens are presented to APC extracellularly, as opposed to intracellular presentation by virus-infected cells, leading to a bias towards antibody-mediated responses. However, many inactivated vaccines directed against viruses, such as human immunodeficiency virus-1 (HIV-1), may not provide a sufficient margin of safety; that is, vaccine candidates free of genetic material. Recombinant technologies have allowed for the production of vehicles with which to deliver vaccines intracellularly (i.e., particle systems, bacterial vectors, and viral-fusion proteins); however, the ability to produce safer avirulent or apathogenic vehicles remains elusive.

3.1. Traditional Vaccine Approaches

Traditional vaccine approaches have focused on immunogens derived from inactivated whole pathogens or components of those pathogens. The rationale is that inactivated pathogens will retain the ability to initiate a specific immune response, but will be unable to elicit a disease response. The majority of vaccines in use are live-attenuated and inactivated or killed vaccines.

Live-attenuated vaccines have the ability to transiently infect a vaccinated individual but are designed to prevent significant adverse effects. Attenuation is achieved by growing the pathogen in non-natural culture conditions and selecting for variants that show reduced pathogenicity. Therefore, these attenuated pathogens will be less capable of growing in a natural environment. Because of the pathogen's limited ability for transient infection in a vaccinated individual, there will be a prolonged exposure to the immune system, allowing for stronger immunity. A disadvantage of live-attenuated vaccines is that there may be a low frequency of reversion of the pathogen to a more virulent stage. Nevertheless, live-attenuated vaccines are good at initiating both humoral and cell-mediated immunity.

The second type of vaccines in use is inactivated or killed vaccines. A whole pathogen is treated either chemically (formaldehyde or various alkylating agents) or physi-

cally (heat, irradiation, ultraviolet [UV]) to inactivate the pathogen. The major benefit with inactivated pathogens is that they can no longer replicate in a vaccinated host. However, many of the methods used to inactivate pathogens could also destroy the epitopes essential for immune-system recognition. Inactivated vaccines are less potent than live-attenuated vaccines because killed viruses can no longer infect cells. Consequently, inactivated vaccines are better at eliciting a humoral response and less effective at generating a cell-mediated response. Under stringent inactivation processes, inactivated pathogens could no longer produce a transient infection in their host, and repeated booster immunizations are necessary to achieve the desired immunity.

3.2. Recombinant Subunit Vaccines

Instead of using the whole pathogen to formulate vaccines, scientists have also made vaccines using purified subcellular macromolecules of a pathogen, usually recombinant proteins. Only one or a limited number of the most important antigens of a pathogen are used in formulating subunit vaccines. Subunit vaccines, free of pathogen genetic material, provide significantly reduced risk of adverse effects. The primary advantage of subunit vaccines is an improved margin of safety because the vaccine is manufactured from recombinant DNA, eliminating the risk of accidental infection owing to viral DNA or RNA. Recombinant proteins can be produced in bacterial or mammalian hosts, allowing for greater yield and purity, better quality control, and lower costs. However, to engineer appropriate subunit vaccines, the key antigenic target of the pathogen must be identified. This can prove to be difficult if a particular pathogen contains a wide spectrum of subspecies or highly variable strains.

Like inactivated or killed vaccines, subunit vaccines are nonreplicative in cells of a vaccinated individual. Therefore, subunit vaccines are also much better at eliciting a humoral response than they are at activating a cell-mediated response. The potential benefits of the improved safety have prompted efforts to develop or improve vaccines using delivery systems and adjuvants that will augment, or enhance, the cell-mediated response to subunit vaccines.

4. Rational Augmentation Strategies

Although a wide spectrum of adjuvants have been developed and demonstrated to enhance the immunogenicity of experimental vaccine antigens, the only adjuvant currently approved by the FDA in the United States for human administration is alum (aluminum salts). Although alum is sufficient to increase antibody response, it is not effective in enhancing cell-mediated immunity. Recent advances in immune recognition mechanisms have allowed for the development of adjuvant designs that can augment cell-mediated responses of antigen. Viral vaccine strategies may benefit from formulation designs that consider two key steps in delivery of antigen to tissues and cells of the immune system. First, antigen must be delivered to target immune tissues where antigens are processed and presented. Second, after antigen has been delivered to these tissues, some degree of success in intracellular delivery to APCs must be achieved to elicit Th cell and effector CTL responses, mimicking natural viral infection in host cells.

The advent and maturation of recombinant DNA technology have provided the ability to readily produce recombinant viral antigens as vaccine candidates. Therefore, if recombinant viral proteins can provide sufficient cellular and humoral responses, their

Table 2
Targeted Delivery of Antigen to Lymphoid Tissues

Carrier	Route of administration	Lymphoid tissue target	References
Liposomes	Intravenous Subcutaneous	Spleen/liver Lymph nodes/ Lymphatic tissues	(13–16,47,49,55,56)
Saponins; ISCOMs	Intravenous Subcutaneous	Spleen/liver Draining lymph nodes	(47,58–60)
Emulsions	Intramuscular or Subcutaneous	Draining lymph nodes	(12,61–65,67–70)
Gene gun	Transcutaneous	Skin	(25,26)
Imiquimod	Transcutaneous	Skin	(21–24)
Toxin-mediated	Transcutaneous	Skin	(17,19,27)
Edible vaccines	Oral	GALT	(17,18,78,79)
Immunoglobulin complexes	Oral/Inhalation	MALT	(35–37)
Microparticles (e.g., PLG)	Oral/Inhalation	MALT	(20,80,81)

potential is tremendous. In addition, adverse vaccine reaction owing to pathogenic reversions encountered in using avirulent virus or bacterial vaccine delivery systems could be avoided. As it is more challenging to elicit cell-mediated responses than humoral responses with recombinant proteins, we will focus our discussion on enhancing cell-mediated responses to viral protein antigens with some experimental delivery systems and adjuvant approaches.

4.1. Strategies to Deliver to Lymphoid Tissues

The first step in delivery of viral vaccine is targeting the vaccine to lymphoid tissues where the antigen can be exposed to cells of the immune system. Diverse organs and tissue are involved in immune presentation throughout the body. The lymph nodes, spleen, cutaneous tissues, and various mucosal-associated lymphoid tissues (MALT) are tissues that trap antigen and allow the antigen to interact with lymphocytes and APCs. Mucosal sites include tonsils, appendix, Peyer's patches in gut-associated lymphoid tissue (GALT), and mucous membranes lining the digestive, respiratory, and urogenital tracts. Targeting or concentrating antigen in these lymphoid tissues will enhance the ability of the immune system to develop cell-mediated responses. Alum-precipitated antigen typically is administered either intramuscularly or subcutaneously and distributes nonspecifically to muscle and other cells in the vicinity of administration and therefore is not very efficient at delivery of antigen to specific tissues of the immune system. Various other delivery systems have been used to increase delivery of antigen to tissues important in immune presentation (Table 2).

Delivery of viral antigen to lymph nodes has been well-studied with several different delivery and adjuvant systems and provides an extremely efficient mode of immune presentation. Emulsions have been shown to concentrate antigen in draining lymph nodes where antigen encounters phagocytic cells of the immune system (12). Liposomes are also extremely efficient in delivering antigen to lymphatic tissues, and the

route of administration influences the ultimate localization of antigen in specific tissues (13–16). After intravenous administration, it is clear that large liposomes are taken up efficiently by macrophages and DCs of the reticuloendothelial system in blood and tissues, including the liver and spleen. Subcutaneously and intramuscularly administered large liposomes may be trapped at injection sites and serve as a drug depot. Small liposomes (50–80 nm) administered subcutaneously will be retained in draining lymph nodes and eventually distribute throughout the lymphatic system and lymphoid tissues (16). Saponins and immunostimulating complexes (ISCOMs) also concentrate antigen in the lymphatic system, perhaps by mechanisms similar to liposomes.

Mucosal immunity, especially in the lungs, intestine, and urogenital sites, is an important first line of defense against infection. Induction of systemic immunity often cannot induce sufficient mucosal immunity; therefore, many researchers have investigated approaches to deliver antigen directly to MALT, which are rich in APCs. Edible vaccines provide a method for oral delivery of viral antigens into Peyer's patches in GALT without the use of additional adjuvants (17,18). Ingestion of transgenic plants expressing viral antigen can provide antigen-specific immunity, and could provide an economical alternative to purified recombinant protein. Genetically modified bacterial enterotoxins, including cholera toxin, heat labile toxin, and pertussis toxin, can induce mucosal immunity, although there are still concerns about safety (17,19). Liposomes, saponins, and microparticles may also be important adjuvants in enhancing mucosal immunity either by oral administration or inhalation into the respiratory tract (17,20). Typically, mucosal vaccines display low immunogenicity and therefore may require higher doses to elicit a response.

Delivery of viral antigens through the skin is an efficient route to eliciting cell-mediated responses. The epidermal layer of skin contains a type of APCs called Langerhans cells, which internalize and present antigen with MHC-II molecules and migrate to lymph nodes where they encounter and activate Th cells. Langerhans cells also stimulate the secretion of IFN- α , interleukin (IL)-12, and IFN- γ , further enhancing cell-mediated responses. A novel method of targeting antigen to cutaneous tissues is with Imiquimod, a topically applied immune response modifier, which activates Langerhans cells. Increases in Th1 and CTL cell-mediated responses have been observed against HSV and human papillomavirus (HPV) when formulated with Imiquimod (21–24). Cell-mediated immune responses have been augmented with other transcutaneous delivery mechanisms such as gene-gun antigen-delivery approaches, which enhance DNA-particle complex penetration across skin cells (25,26), and delivery of antigen and enterotoxin via an adhesive patch (27).

Increased understanding of mechanisms involved in immune responses has brought about new ways for researchers to think about designing and targeting more specific and effective vaccines, so called "virtual pathogens" (28). By combining various elements that are known to induce or regulate cell-mediated responses, researchers could generate prototypic viral vaccines that will mimic natural infection. These viral vaccines potentially could deliver subunit vaccines containing viral antigen epitopes, known to initiate immune responses, and cytokines, known to regulate the responses, in a lipid bilayer. Other molecules can be added to further enhance immune responses, including mannose receptors targeting APC receptors important for antigen uptake and processing, and prokaryotic signals such as CpG motifs, which will also activate APCs. Virtual pathogen vaccines may hold promise for incorporating the immunogenic properties of a virus without causing pathogenicity.

Table 3
Delivery Vehicles for Intracellular Targeting

Delivery vehicles	Examples	Viral protein antigen	References
Particles	Liposomes	HIV; HSV; influenza; hepatitis A; hepatitis B; adenovirus; rabies; measles; rubella	(14,15,48,50–54)
	Saponins; ISCOMs	HIV; SIV; influenza; HSV; hepatitis C; hepatitis B	(82–93)
	Emulsions	HSV; influenza; HIV; CMV; hepatitis B; SIV	(63–65,67–70,94)
	Microparticles	HIV; SIV; hepatitis B; Influenza	(19,20,80,81,95)
Viral vectors	Vaccinia virus	HIV; HSV; rabies; Newcastle disease; Japanese encephalitis	(96,97)
	Adenovirus	hepatitis B; rabies	(98,99)
	Alphavirus	HIV; SIV; influenza	(100,101)
	Adeno-associated virus	HSV	(102–104)
	Poliovirus	SIV	(105)
	Yellow Fever virus	West Nile virus	(106)
Bacterial vectors	<i>Salmonella</i>	HSV; influenza; lymphocytic choriomeningitis virus	(107,108)
	<i>Listeria monocytogenes</i>	HIV; influenza; lymphocytic choriomeningitis virus	(109,110)
	<i>Vibrio cholera</i>	HIV; rotavirus	(108,111)

4.2. Delivery Strategies for Intracellular Targeting

After delivery of viral antigen to lymphoid tissues, the next issue in augmenting cell-mediated immunity is intracellular delivery. Several delivery systems can target viral antigen to intracellular compartments of cells, necessary for MHC-I processing and presentation and CTL responses. Some of the approaches, including viral and bacterial vectors, designed for intracellular delivery are summarized in Table 3. Live vectors are quite efficient at mediating intracellular delivery of antigen; this issue has been extensively reviewed (29–31). Hence, we will focus on nonviral approaches, free of genetic material, to target viral antigens intracellularly.

Particulate carriers such as liposomes, ISCOMs, emulsions, and microparticles efficiently deliver viral antigen into the cytosol of cells, mimicking natural infection, to elicit a cell-mediated response (19,32). The dimensions of these particles, similar to that of infecting microorganisms, allow for phagocytosis and some degree of success in delivery of antigen of APCs for presentation by both MHC-I and MHC-II molecules. These vaccine carriers may also induce cytokine secretion, further augmenting cell-mediated responses.

Entrapment of antigens in biodegradable microparticles may enhance intracellular delivery as well as lymphoid tissues targeting. Poly(lactide-co-glycolide) (PLG) polymers and other polymeric materials are especially efficient in delivery of antigen to mucosal tissues (17,19,20). When delivered orally, PLG polymers are taken up, with some degree of preference, by cells in Peyer's patches of the lower intestine where enriched populations of APCs are located. Intranasal administration of PLG polymers

leads to concentration of antigen in draining lymph nodes. In addition, PLG polymers fabricated as microparticles may enter the cytosol of epithelial cells on the mucosal surface, allowing for presentation by MHC-I molecules eliciting a cell-mediated response.

Several other techniques are also under investigation to enhance intracellular delivery. Modified liposomes, called cochleate delivery vehicles, are multilayered structures composed of a lipid bilayer sheet rolled up or stacked in sheets with no internal aqueous space (33). Antigens incorporated within the interior of the cochleate structure are protected from degradation. Cochleates fuse with cell membranes and deliver antigens into the cell. The use of lipopeptides is another vaccine strategy that may provide enhanced intracellular delivery of antigens without the need for additional adjuvants (34). Lipopeptides are composed of antigen covalently linked to lipid moieties, which may help penetrate the cell membrane of APCs, allowing rapid intracellular delivery of the antigen. Immunotargeting of antigen, fused to an Fc fragment, may also enhance antigen uptake into APCs (35–37). The antigen-Fc complex binds Fc receptors on dendritic cells, is internalized and processed, and is subject to MHC-I presentation.

Enhanced cellular uptake of antigen could be achieved with immunostimulatory oligonucleotides containing cytosine-phosphate-guanosine (CpG) motifs. These motifs are found in bacterial DNA and have been shown to induce multiple immune responses. CpG motifs are composed of approx 20 unmethylated cytosine and guanine dinucleotides, usually with two 5' purines and two 3' pyrimidines at either end (38). CpG motifs are recognized as common features of an infectious agent by cells of the innate immune system through the Toll-like receptor (TLR) 9 (39). Binding of the CpG DNA to the TLR initiates endocytosis and induces macrophages and DCs to secrete cytokines, which induces NK cells and aids in Th1 differentiation and enhancement of CTL levels (40–42). CpG motifs may also induce secretion of a broad range of cytokines that may further enhance immune responses. The cell-mediated responses elicited by CpG motifs appear to be enhanced by attachment to protein antigens and formulation with other delivery systems such as alum, liposomes, and emulsions (19,43). CpG oligonucleotides have been shown to induce humoral and cell-mediated responses, either alone or in combination with alum or incomplete Freund's adjuvant, to a variety of viral pathogens, including hepatitis B (44,45), HSV-1 and -2 (46), and HIV-1 (41).

In the following, we will discuss in some detail some the mechanisms of vaccine delivery systems for protein antigens designed to augment cell-mediated responses. We will also discuss the role administered cytokines play in enhancement and regulation of immune responses.

4.2.1. Liposomes Mediate Tissue and Intracellular Delivery

Liposomes are colloidal particles composed of phospholipid molecules in the formation of an enclosed lipid bilayer. Soluble antigens can be enclosed in the internal aqueous space or amphipathic antigens can incorporate in the lipid bilayer. Liposomes have been used to enhance antigen-specific immune responses for various vaccines. The influence of physiochemical properties of liposomes on the antigen immune response, such as size, charge, membrane fluidity, and epitope density, has been studied extensively (47). Liposomes deliver encapsulated antigen both to lymphoid tissues for antigen presentation and intracellularly to elicit cell-mediated responses. As discussed earlier, the route of administration of liposomes is important in delivering antigen to appropriate tissues. Once in lymphatic tissues, liposomes will be phagocytosed and

liposome encapsulated antigen will be processed and presented by APCs. In addition to macrophages that predominantly present antigen with MHC-II molecules, antigen delivered to some endothelial, Langerhans, and dendritic cells is more likely to be presented by MHC-I molecules, therefore mediating cellular immune responses, including CTL activation (48).

Liposomes also can deliver antigen intracellularly by fusing with the plasma membrane to deliver antigen into the cytoplasm of APCs to induce a cell-mediated response. Therefore, liposome formulation may provide an excellent way to enhance antigen delivery and presentation to stimulate both humoral and cell-mediated immune response to vaccines. The efficiency of intracellular delivery will depend on the composition of liposome membranes and the inclusion of bio-sensors to amplify delivery. For example, listeriolysin O (LLO) is a purified protein from the cytosol-invading bacterium, *Listeria monocytogenes*, that enhances intracellular delivery of antigens when encapsulated in liposomal carriers. LLO has been shown to deliver ovalbumin into the cytosol of APCs; however, the technology will allow for other antigens, such as viral peptides, to be introduced in this manner (49). Researches have shown that subcutaneous immunizations of LLO-containing liposomes generated stronger CTL responses than intravenous immunizations, consistent with our hypothesis that subcutaneous injection directs liposomes to draining lymph nodes, and eventually the entire lymphatic system, for antigen presentation. Immune stimulators have also been incorporated into liposomes to increase their stimulation of the immune system. When the lipophilic adjuvant muramyl tripeptidyl-phosphatidylethanolamine (MTP-PE) is in liposome formulation with HIV-envelope protein, a dramatic increase is observed in humoral and cell-mediated responses (50) or HSV-1 and -2 antigens (51–54). Liposomes can also be actively targeted to specific cells within tissues to enhance the immune response (16). A variety of ligands or receptors unique to the target cells have been formulated in the liposome membrane, including antibodies, growth factors, cytokines, hormones, and toxins (16).

Another method to increase intracellular delivery of viral antigens is to use specialized liposomes designed with viral membrane proteins, called virosomes. Virosomes enhance intracellular delivery by utilizing targeting and fusogenic properties of viruses (19). Two new virosome-based recombinant protein vaccines currently on the market incorporate influenza virus surface glycoproteins into the lipid bilayer to mediate intracellular delivery of viral antigens. The first virosome vaccine approved for human use was a hepatitis A vaccine (Epaxal[®]) containing liposome-encapsulated formalin-inactivated hepatitis A virus particles (55). The advantage of Epaxal is that the immune response is enhanced for both hepatitis A antigen and influenza virus antigen. A second virosome-based influenza vaccine is Inflflexal V[®], a trivalent product that generates enhanced immunogenicity compared with conventional influenza vaccines (56).

4.2.2. Saponins Provide Intracellular Delivery of Antigen

Saponins are compounds with potent immunological activity purified from *Quillaja saponaria*, a South American soap-bark tree. Saponins, which form micellar suspensions, have been shown to have a strong adjuvant effect on antibody and cell-mediated responses. The commonly used saponin Quil A is actually a partially purified mixture of various saponins from crude extracts of *Quillaja saponaria*. Further purifications of Quil A have provided purified saponins with potent adjuvant effects such as QS-21 and QS-7,

which may also produce less toxicity than Quil A. QS-21 has been shown to enhance immune responses to gp120 HIV-1_{MN} envelope protein in human immunizations, and may allow for the administration of a lower dose of antigen compared to alum (57).

A unique feature of saponins is their strong affinity for cholesterol; this ability is exploited in the formulation of ISCOMs, which are spherical, colloidal particles composed of saponins (typically Quil A), cholesterol, phospholipids, and antigen (47,58). ISCOMs are composed of regularly ordered micellar subunits that form the shape of a ring. ISCOM formulation with antigenic proteins is more restricted than formulation of antigens with liposomes. Although amphipathic proteins are usually easily incorporated into ISCOMs, extremely hydrophobic, large, and soluble proteins may be harder to formulate. The adjuvant effect of ISCOMs formulated with Quil A has been shown to be greater than the effect of Quil A alone. The mechanisms by which Quil A and ISCOMs elicit an enhanced immune response are not entirely understood; however, they appear to work in a similar fashion. Quil A and ISCOMs cause a local inflammatory response, which recruits lymphocytes and macrophages important in antigen presentation. Various saponins may stimulate T and B cells differently and to various degrees (59). Like liposomes, ISCOMs and saponins strongly enhance CTL responses, perhaps by fusion with the cell membrane of APCs (47). ISCOMs are able to generate a number of immune responses, including APC activation, secretion of IL-2 and IFN- γ , and the induction of Th cell and CTL responses (60).

4.2.3. Emulsions Target Antigen to Lymph Nodes

Oil-in-water emulsions have been shown to induce both humoral and cellular immune responses to subcellular or subunit vaccines. Emulsions are made through a microfluidization process that creates small oil droplets formulated with antigen in an aqueous solution. A mechanism of action for emulsions may be the depot effect, in which emulsions facilitate the transport of antigen to the lymph nodes (12). Emulsions may also interact with cell membranes in a manner similar to liposomes. Two prototypical emulsion adjuvants that have been studied to enhance the immune response are complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA); however, both have been found to exhibit toxicity and have not been approved for human use. Freund's adjuvants stimulate a local inflammatory response at the site of injection, which attracts both macrophages and lymphocytes. IFA, composed of antigen in an aqueous solution, mineral oil, and an emulsifying agent, allows for the antigen to be released slowly from the injection site. CFA is similar to IFA, except that it contains immunostimulatory mycobacterial cell-wall components, such as muramyl dipeptide in the emulsion that makes CFA more potent than the incomplete form. However, the inclusion of bacterial elements in CFA increases the potential for toxicity. A less toxic oil-in-water emulsion, Adjuvant 65, has been used as an adjuvant but is also not approved for human administration (61,62).

Two of the new safer approaches to using emulsions as a modifier of immune responses are MF59 and Syntex Adjuvant Formulation-m (SAF-m). MF59, a squalene-in-water emulsion, has been shown to augment cell-mediated responses to HSV (63), influenza (63), HIV-1 (64), and cytomegalovirus (CMV) (65) subunit vaccines, and can also be formulated with the immunostimulator MTP-PE, which provides further enhancement. The release rate of MTP-PE from MF59 is higher than from liposomes, therefore increasing the risk of severe local and systemic reactogenicity (66). Regardless, MF59

appears to be internalized by DCs, which function as APCs and activate mechanisms of cell-mediated responses (12). SAF-m is another squalene-in-water emulsion used as an adjuvant to enhance cell-mediated immunity to a variety of viruses, including hepatitis B (67), influenza (68), simian immunodeficiency (SIV) (69), and HSV (70). However, some of the newer emulsions may also have significant adverse effects.

4.2.4. Cytokines Play a Role in Regulating and Enhancing Immune Responses

Cytokines are an important additional consideration in enhancing cell-mediated responses to subunit vaccines. Cytokines are a group of small proteins important in cell-to-cell communications that influence immune interactions throughout the body. The biological activities of cytokines are often mediated by multiple receptors expressed on cell surfaces. Cytokines can generally be divided into two categories: colony-stimulating factors (CSF) and lymphokines. Colony-stimulating factors, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM-CSF), and monocyte CSF (M-CSF), are large glycoproteins produced primarily by APCs to stimulate the growth of immune progenitor cells. Lymphokines, such as ILs, are produced by leukocytes and stimulate leukocyte development and the production of IFNs (71).

Cytokines regulate the immune system by stimulating or inhibiting the activation and differentiation of various cells and by regulating the secretion of antibodies and other cytokines. Cytokines may modulate antigen presentation by activating APCs and increasing the number of activated APCs. APCs express numerous cytokine receptors on their cell surface, and binding of a cytokine to its receptor results in activation, migration, and maturation (28). Cytokines also regulate B cell proliferation and differentiation into antibody-secreting cells. Once antigen is presented to T cells by APCs, cytokines are important in enhancing cell-mediated immunity by regulating the clonal expansion of antigen-specific T cells into Th cells and CTLs.

A number of laboratories have investigated the ability of interleukins to enhance cell-mediated immunity. IL-2 has been shown to influence multiple immune functions in addition to its ability to induce T-cell proliferation. IL-2 stimulates the growth of both cytotoxic and Th cells as well as B cells, macrophages, and NK cells (72). Mice vaccinated with a fusion protein composed of HSV glycoprotein D (gD) and human IL-2, displayed complete immunity against HSV challenge, whereas those vaccinated with gD formulated in alum showed no such protection (73). IL-2 and IL-7 also produced similar protection against HSV infection through enhancement of CTL responses (74). IL-2 may also have clinical relevance in HIV infection. Studies have shown that IL-2 therapy in HIV patients, in combination with highly active antiretroviral therapy (HAART), increases CD4 and CD8 cell counts, decreases HIV-1 latently infected CD4 cells, and decreases viral load in peripheral blood mononuclear cells (PBMCs) compared to HAART alone (75).

A second interleukin, IL-12, may also be important in enhancing cell-mediated immune responses to viruses. The primary functions of IL-12 are promoting Th1 and NK cell growth and secretion of IFN- γ , and activation of CTLs. Cell-mediated immunity enhancement by IL-12 may also be important in HIV vaccine and therapy; some approaches using IL-12 have been reviewed (76). IL-12 has also been shown to work synergistically with GM-CSF and TNF- α to increase CTL response to HIV-1 antigen. An HIV vaccine trial in nonhuman primates found that IL-12 also stimulates both humoral and cell-mediated immune responses to HIV-1 envelope protein gp120 (77).

Cytokines are the messengers of the immune system and generally act locally on cells in close proximity to the producer cell. Consequently, cytokines have short half-lives and systemic concentrations are low. The high systemic levels of cytokines often administered to induce cell-mediated immunity to virus are frequently associated with adverse effects. Therefore, the toxicity and short half-life of cytokines *in vivo* has limited their effectiveness as an adjuvant (28). Targeted delivery of cytokines could provide sufficient concentrations at sites of antigen presentation and expansion of B and T cells while limiting systemic cytokine levels. When successfully delivered at the site of action, cytokines may provide cell-mediated immune enhancement not achieved by increased delivery of antigen alone. Therefore, incorporating cytokines into other targeted delivery systems and adjuvants may enhance their ability to elicit and regulate cell-mediated responses.

5. Summary

Cell-mediated responses that can eliminate virus-infected cells are important for combating viral infections. Mechanisms of both innate and adaptive immunity play a role in cell-mediated responses to viral infection, but the activation of antigen-specific CTL responses is critical for complete viral clearance from the body. Advances in recombinant protein technologies have allowed for the development of subunit vaccines. These recombinant vaccines are safer than traditional vaccines but tend to elicit humoral rather than cell-mediated immunity. Several vaccine delivery and adjuvant strategies discussed in this chapter could provide significant augmentation of cell-mediated immune responses to viral antigens, thus providing a greater degree of protection. The two important issues in enhancing cell-mediated responses to viruses are targeting viral antigens and cytokines to immune tissues central to proper antigen presentation, and efficient intracellular delivery of antigen to APCs. It is likely that one or more of these vaccine and cytokine delivery systems and adjuvants will provide breakthroughs in recombinant vaccines capable of inducing both humoral and cell-mediated responses.

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