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Mucosal SIgA Enhancement: Development of Safe and Effective Mucosal Adjuvants and Mucosal Antigen Delivery Vehicles

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14.1. Introduction

14.1.1 Unique Aspects of the Induction and Regulation of IgA Antibody Responses

The respiratory and digestive tracts represent major entry routes for pathogenic microorganisms from the lumen into an almost sterile environment of the body. Several physical and biological barriers associated with the innate immune system protect these sites from invasion and help to maintain mucosal homeostasis. The first physical defense line is a barrier structure made up of epithelial cells (ECs) joined firmly by tight junction proteins with brush-border microvilli and a dense layer of mucin (Berkes et al., 2003). Antimicrobial peptides such as defensins and type II phospholipase A2 produced by ECs and Paneth cells are additional molecules preventing the attachment and penetration of pathogenic microorganisms into mucosal tissues (Selsted and Ouellette, 2005).

In addition to these physical and biological barriers, immunological barriers, most notably secretory immunoglobulin A (SIgA) antibody (Ab), which is the predominant isotype at mucosal sites, play an important role in preventing invasion by pathogens (Kunisawa and Kiyono, 2005). A distinct feature of SIgA when compared with IgG is the ability to form polymers [polymeric IgA (pIgA)], mediated by the J-chain produced by mucosal plasma cells (Halpern and Koshland, 1970; Mestecky et al., 1971; Woof and Mestecky, 2005). The pIgA requires cooperation with mucosal ECs expressing the polymeric Ig receptor (pIgR) for transport into the lumen (Kaetzel, 2005; Kaetzel et al., 1991) (see Chapter 3). The pIgR is expressed on the basal membrane of ECs and acts as a receptor for the mucosal pIgA containing the J-chain, thereby accelerating the internalization and transport of the complex to the apical site via transcytosis. Cleavage of pIgR to the secretory component (SC) at the apical surface releases SIgA into the lumen. These SIgA Abs abolish microbial infections by inhibiting their adherence to host ECs. SIgA Abs also neutralize exotoxins by binding to their biologically active site. Thus, SIgA seems to be the sole immunological molecule exhibiting antigen specificity in the outside regions of our body. Furthermore, the SC moiety of SIgA confers novel functions that enhance mucosal immune defense (see Chapter 8).

14.1.2. Common Mucosal Immune System - Dependent IgA Induction Pathways

To induce antigen-specific IgA via mucosal sites, mucosal tissues contain a mucosal network known as the common mucosal immune system (CMIS) linking inductive and effector tissues (Fig. 14.1) (Kunisawa and Kiyono, 2005). The major inductive site for orally administered antigen is the gut-associated lymphoreticular tissues (GALT), which include the Peyer's patches (PPs), and for nasally administered antigen, it is the nasopharynx-associated lymphoreticular tissue (NALT) (Kiyono and Fukuyama, 2004; Kunisawa et al., 2005) (see Chapter 2). As an additional inductive tissue, isolated lymphoid follicles (ILFs) were identified throughout the intestine (Hamada et al., 2002). In spite of differences in their organogenesis pathway, the inductive tissues share several features for initiation of antigen-specific immune responses (Kiyono and Fukuyama, 2004; Kunisawa *et al*., 2005). For instance, the PPs and NALT are overlaid by a follicle-associated epithelium (FAE) containing antigen-sampling M (microfold)-cells for selective antigen uptake into underlying regions containing antigen-presenting cells (APCs) such as dendritic cells (DCs) (Neutra et al., 2001). The immunological interactions among DCs, T-cells, and B-cells in these inductive tissues promotes IgA commitment of B-cells, which undergo a μ-to-α isotype class-switch recombination in the germinal centers of the inductive tissues (Brandtzaeg and Johansen, 2005; Shikina et al., 2004). In contrast to the dominant class switch to IgA in PPs, B-cell differentiation in NALT leads to the production of both IgA and IgG following sequential class switch from Cμ to Cα via Cγ (Shimoda et al., 2001). These findings may explain the equal commitment of B-cells to IgG and to IgA in NALT, but further analyses will be required to reveal the molecular mechanisms involved in the generation of mucosal B-cells that express those two different isotypes.

Following the class switch to IgA by mucosal B-cells through their interaction with T-cells and APCs, both B- and T-cells emigrate from the inductive tissue (e.g., PPs and NALT), circulate through the bloodstream, and home to distant mucosal effector compartments, especially the lamina propria (LP) regions of the gastrointestinal (GI), respiratory, and reproductive tracts (Fig. 14.1). Although immunization via one mucosal site often activates other, remote mucosal sites, immunization via certain mucosal inductive tissues can lead to the preferential induction of humoral immune responses in the same mucosal sites. The tropism of B- and T-cells is determined by the site-specific combination of adhesion molecules and chemokines. Several lines of evidence have suggested that intestinal DCs play a crucial role in determining the gut tropism of T-cells (Iwata et al., 2004Johansson-Lindbom et al., 2003; Mora et al., 2003; Stagg et al., 2002). They induce $\alpha_4\beta_7$ integrin and CCR9 on antigen-primed T-cells, which interact with the mucosal addressin cellular adhesion molecule-1 (MAdCAM-1), expressed by the endothelium in the LP, and the thymus-expressed chemokine (TECK, also known as CCL25), produced by small-intestinalECs, respectively, which determines the gut-tropism of T-cells. A similar pathway is now proposed for B-cells. On the other hand,

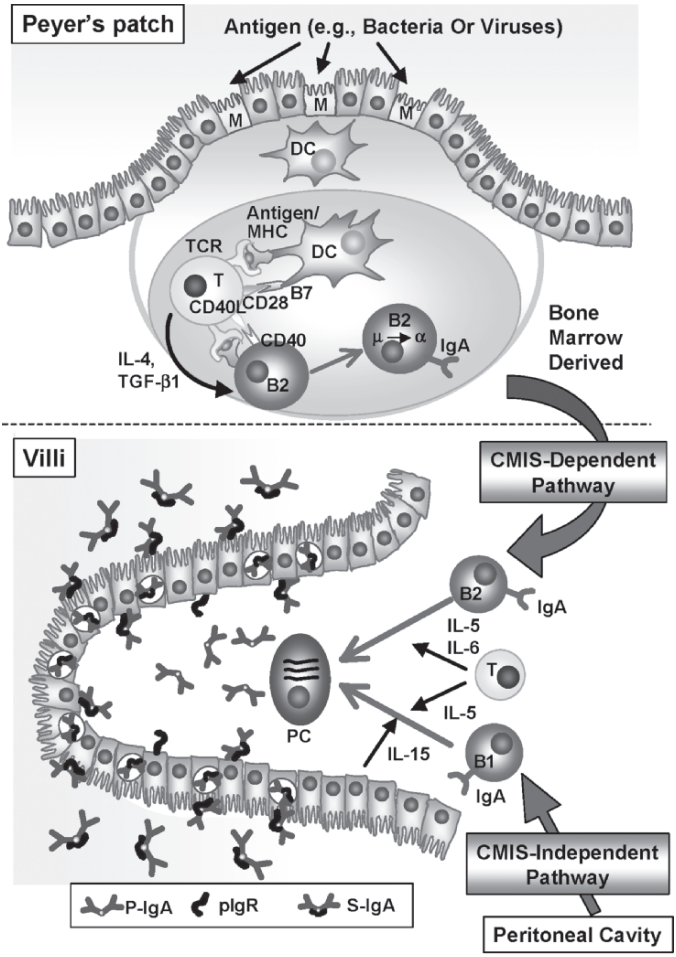

FIG. 14.1. CMIS-dependent and CMIS-independent pathways for the induction of SIgA Ab responses. In the CMIS-dependent pathway, M-cells take up the luminal antigen for transport to the DCs, which, in turn, activate naïve B-cells, also known as B2-lymphocytes, in a T-cell-dependent manner within PPs. In this step, several factors (e.g., CD40, cytokines) induce class-switch recombination from IgM to IgA. The IgA-committed B-cells exit through the lymph and home through the thoracic duct and peripheral blood to mucosal effector sites like the lamina propria of the GI tract. In these effector sites, IgA-committed B2-cells are stimulated by cytokines, including IL-5 and IL-6, resulting in their differentiation into plasma cells (PCs). The plasma cells produce IgA as a dimer joined by the J-chain. Dimeric or higher-molecular-weight forms of p-IgA bind to the pIgR on epithelial cells and are transported across the epithelium and released into the intestinal lumen as SIgA. Another lineage of B-cells, B1-cells, are mainly derived from the peritoneal cavity and act as a CMIS-independent source of intestinal SIgA.

nasal immunization induces the upregulation of $\alpha_4\beta_1$ integrin and CCR10, allowing selective trafficking of B-cells to nasal passage epithelium expressing their ligands, VCAM-1 and CCL28, respectively (Kunkel et al., 2003).

Upon arrival at effector sites, such as the LP in the aerodigestive tracts, the IgA-committed B-cells further differentiate into IgA-producing plasma cells under the influence of interleukin (IL)-5, IL-6, and IL-10 (Hiroi et al., 1999; McGhee et al., 1991; Takatsu et al., 1988). In addition to the help provided by the IgA-enhancing cytokines, IgA production into the lumen requires the expression of the J-chain and pIgR, as mentioned earlier. Along with SIgA, mucosal cytotoxic T-lymphocyte (CTL) responses are important for the clearance of enteric or respiratory viruses and intracellular parasites, such as *Listeria* (Shastri et al., 2005). In this respect, like the IgA-producing B-cells, large numbers of mucosal T-cells are continuously induced by the CMIS-dependent pathway.

14.1.3. CMIS-Independent SIgA Induction Pathways

In addition to the CMIS-dependent pathway, several CMIS-independent pathways have also been identified, especially in the GI tract. It was shown that antigen-specific IgA Ab responses can be induced even under conditions of PP deficiency and thus suggested the presence of additional antigen sampling sites for the induction of antigen-specific immune responses (Yamamoto et al., 2000; Kunisawa et al., 2002). As an alternative gatekeeper in the GI tract, we have identified M-cells on villi (we termed them villous M-cells) that are capable of taking up antigen from the intestinal lumen (Jang et al., 2004). Intestinal villous M-cells develop in various organized lymphoid tissue-defective (e.g., PP-/ILF-null) mice and are capable of taking up bacterial antigens. The discovery of villous M-cells has not only shed light on a novel gateway for antigen uptake into the GI tract but has also suggested the possibility of previously unsuspected routes of pathogen invasion.

In addition to villous M-cells, recent studies have now identified a unique DC population among intestinal ECs (Nies*s et al.,* 2005; Rescigno et al., 2001). These intraepithelial DCs migrate into the epithelium via a CX3CR1-mediated pathway and express tight-junction-associated proteins (e.g., occuludin, claudin 1, and zona occuludens 1) for extending their dendrites between ECs and taking up gut luminal antigens.

Unique subsets of B-cells are also found in the intestine. Two lineages of murine B-cells, B1- and B2-cells, can be distinguished by differential expression of cell surface molecules (B220, IgM, IgD, CD5, and Mac-1), origin, growth properties, and antigen-specificity (Berland and Wortis, 2002). "Conventional" B2-cells originate from the bone marrow, recognize primarily T-dependent protein antigens, and are capable of undergoing affinity maturation and memory cell generation in mucosal inductive sites (see Chapter 2). Some of the mucosal IgA plasma cells are derived from B2-cells, which home to mucosal sites via the CMIS-mediated pathway. In contrast, B1-cells are self-renewing and recognize primarily T-independent nonprotein antigens with low affinity. Significant numbers of B1-cells are found in the pleural and peritoneal cavities, as well as

mucosal effector sites like the LP of the aerodigestive tract. IL-5, a well-known IgA-enhancing cytokine, was shown to induce the differentiation of both B1- and B2-cells into IgA plasma cells (Hiroi et al., 1999). In contrast, mucosal EC-derived IL-15 promoted IgA commitment and differentiation of B1-cells but not B2-cells (Hiroi et al., 2000). Recent evidence has suggested that intestinal B1-cells migrate from noninductive sites, presumably the peritoneal cavity, to effector sites. Examination of B-cell populations in *aly/aly* mice, which carry a point mutation in the nuclear factor (NF)-κB-inducing kinase (NIK), demonstrated elevated B-cell levels in the peritoneal cavity but a complete absence of B-cells in the LP of the GI tract (Fagarasan et al., 2000). Those findings suggested that NIK-mediated signals are essential for the migration of B1-cells into the intestinal compartments.

Because B1-cells respond to T-cell-independent antigens, SIgA Ab production originating from B1-cells was detected in TCR β- and δ-chain-deficient mice (Macpherson et al., 2000). In another study, about 65% of fecal bacteria bound B1-derived SIgA Abs, whereas 30% of bacteria bound B2-derived SIgA, indicating that B1-cell-derived SIgA Abs recognized a large population of commensal bacteria (Bos et al., 2000). Thus, it is likely that the B1-cell-derived SIgA not only inhibits pathogenic bacterial invasion but also plays an important role in maintaining mucosal homeostasis by preventing the attachment of commensal bacteria to mucosal epithelial cells. In contrast, B2-cell-derived SIgA is a key protective Ab against pathogenic microorganisms induced in a T-cell-dependent manner.

In addition to B-cells, a unique subset of T-cells also characterizes the CMIS-independent mucosal immunity. This T-cell population is usually referred to as intraepithelial lymphocytes (IELs). The IELs occur at a frequency of 1 IEL/4–9 ECs and express either TCRαβ or TCRγδ. Thus, it has been suggested that IELs are the bridge between innate and acquired immunity. The IEL function and characteristics have been well reviewed elsewhere (Cheroutre, 2005; Guy-Grand and Vassalli, 2002).

14.2.2. Why are Mucosal Adjuvants Essential for the Induction of IgA Antibody Responses?

The fascinating characteristics of the mucosal immune system in the prevention of infections by pathogens has led to much attention for the development of mucosal (e.g., oral and nasal) vaccines. Mucosal vaccines offer numerous advantages over traditional injection-type parenteral vaccines, including needle-free, easy administration and the possibility of self-delivery. Most importantly, mucosal vaccines can induce both mucosal and systemic immune responses, whereas parenteral immunization yields only systemic immune responses. Hence, traditional parenteral immunization does not lead to the generation of mucosal immunity which would inhibit the initial attachment of pathogens to host cells in mucosal sites.

In contrast, mucosal vaccines can establish a first line of immunological defense at mucosal sites as well as provide a systemic immune surveillance to detect and destroy invading pathogens.

In general, it is often difficult to induce strong SIgA Ab responses as well as T-cell-mediated immune responses at mucosal and systemic sites by mucosal immunization with protein antigen alone. Subunit protein-based vaccines are generally safer than live attenuated or inactivated vaccine using whole microorganisms, but they are less efficacious because of the potential for degradation in the aerodigestive tracts under conditions of low pH, numerous digestive enzymes, and detergent activity by bile salts. Other problems with delivery of protein-based vaccines to mucosal inductive sites include significant dilution in the lumen and physical barriers, including mucus and epithelial tight junctions. The same issues also apply to mucosal DNA or RNA vaccines.

Another major problem in the development of mucosal vaccines is the potential for development of oral or nasal tolerance instead of SIgA Ab-mediated mucosal immunity. Both oral and nasal tolerances are naturally achieved in order to prevent or suppress the development of harmful immune responses against ingested or inhaled foreign proteins. Generally, orally or nasally administered antigen induces immunological tolerance by the induction of T-cell deletion, T-cell anergy, or regulatory T-cells, which are determined by the dose of antigen given (Dubois et al., 2005).

To overcome these obstacles in the development of mucosal vaccines, major efforts are being aimed at development of mucosal adjuvants as well as antigen delivery systems. At a minimum, these systems should protect the antigen from physical and biological elimination. In addition, a major research focus has been aimed at molecular and cellular elucidation of key immunological mechanisms for the simultaneous induction and regulation of active (e.g., SIgA) and silent (e.g., tolerance) immune responses. In this chapter, we will discuss the recent advances in the development of mucosal adjuvants and antigen delivery systems for use with prospective mucosal vaccines for successful SIgA Ab responses.

14.3. Enterotoxin-Based Mucosal Adjuvants

14.3.1. Cholera Toxin and the Escherichia coli Heat-Labile Enterotoxin Are Potent Enhancers of Mucosal SIgA Production

Perhaps the most potent mucosal adjuvants are the bacterial toxin derivatives. Among them, cholera toxin (CT) and the closely related heat-labile enterotoxin (LT) are the most effective and well-studied mucosal adjuvants, which are derived from *Vibrio cholerae* and *Escherichia coli*, respectively (Lycke, 2005; Rappuoli et al., 1999; Yamamoto et al., 2001). They are not only potent

immunogens but also adjuvants that enhance both mucosal and systemic immune responses against mucosally coadministered antigens. These enterotoxins possess adjuvant activity when given by oral, nasal, or rectal routes, and the antigen should be administered by the same route as the adjuvant (Lycke and Holmgren, 1986). These findings indicate that local molecular and cellular interactions among the adjuvant, antigen, and the host mucosal immune system are all essential for maximal adjuvanticity.

Cholera toxin and LT are structurally similar (83% homology at the amino acid level) hexameric toxins composed of one A subunit (CT-A or LT-A) and a pentamer of B subunits (CT-B_5 or LT-B_5) (Spangler, 1992). The A subunit contains two distinct domains, A1 and A2, which are linked by a disulfide bond. The A1 domain possesses ADP-ribosyltransferase activity and the A2 subunit is responsible for linking the A1 with the B subunit (Spangler, 1992). The B subunit participates in the binding of CT or LT to host cells. Different binding activities between CT-B and LT-B have been reported. The CT-B binds to GM1-ganglioside, whereas the LT-B binds to GM1-ganglioside as well as asialo GM1 and GM2 (Fukuta et al., 1988; van Heyningen, 1977). They also exhibit different immunological effects. For instance, CT shows a bias for inducing IL-4-mediated Th2-type responses, whereas LT induces both Th1- and Th2-type responses associated with interferon (IFN)-γ, IL-5, IL-6, and IL-10 production (Takahashi et al., 1996; Xu-Amano et al., 1993; Yamamoto et al., 1999). In this regard, it was reported that CT inhibited IL-12 production and alternatively induced IL-10 by DCs, macrophages, and ECs, which might account for the dominant induction of Th2-type responses by CT (Braun et al., 1999; Soriani et al., 2002). Further implication of the Th1- and Th2-inducing ability of the toxins was obtained from experiments using artificially created chimeric forms of the enterotoxin adjuvants. To address which subunit plays a crucial role in determining the balance of Th1 or Th2 response, chimeras of CT-A/LT-B and LT-A/CT-B were constructed (Bowman and Clements, 2001; Boyaka et al., 2003; Kweon et al., 2002). Both chimeras induced SIgA Ab responses, and, intriguingly, CT-A/LT-B upregulated both Th1- and Th2-type responses, wherease LT-A/CT-B promoted only Th2 responses. These observations imply that the B subunit of a particular enterotoxin adjuvant is mainly responsible for their propensity to direct Th cell subset responses.

For cellular aspects of enterotoxin-induced adjuvanticity, several possible pathways operate via different subsets of immunocompetent cells. For example, both CT and LT trigger APCs such as DCs. As mentioned earlier, DCs play a pivotal role in the initiation of antigen-specific immune responses in the inductive tissues like PPs (Sato and Iwasaki, 2005). It was previously reported that CT induced migration of DCs from the subepithelial dome to T- and B-cell areas of PPs, which resulted in efficient interactions among DCs, T-cells, and B-cells (Shreedhar et al., 2003). CT additionally induced the maturation of DCs accompanied by the upregulation of major histocompatiblity complex (MHC) and costimulatory molecules (CD80 and CD86), thereby promoting potent T- and B-cell responses (Cong et al., 1997;

Yamamoto et al., 1999). CT has also been shown to promote the production of IL-1, IL-6, and IL-10 from both DCs and macrophages (Cong et al., 2001). The direct effects of CT and LT as adjuvants are not only specific for APCs, but also for the other immunocompetent cells. Binding of LT to B-cells induced upregulation of MHC class II and CD25, which was associated with the activation of extracellular signal-regulated kinase (Erk1 and Erk2) (Bone et al., 2002). In addition, CT enhances B-cell isotype switching by two different mechanisms (Lycke, 1993). In the first case, ADP-ribosyltransferase activity for cAMP induction was involved in the germline IgH-chain RNA transcripts. In the second case, the binding of CT-B to the GM1-ganglioside on B-cells promoted B-cell differentiation (Lycke, 1993). Enterotoxins also directly activate T-cells, especially $CD4^+$ T-cells (Williams et al., 1999). Thus, the adjuvant activity of CT was shown to be decreased in CD4-deficient mice (Hornquist et al., 1996).

In addition to SIgA and plasma IgG Ab responses, CTL responses are also primed by oral immunization with CT or LT (Bowen et al., 1994; Simmons et al., 1999). For the induction of CTL responses, antigen processing in the cytoplasm and endoplasmic reticulum (ER) and subsequent MHC class I-restricted antigen presentation are required (Kunisawa and Shastri, 2003; Shastri et al., 2005). In this regard, molecular studies concerning intracellular CT trafficking revealed a unique transport pathway for CT in antigen delivery (Fig. 14.2) (Lencer and Tsai, 2003). Following CT binding to the host GM1 ganglioside through CT-B, CT is internalized by both clathrin-dependent and clathrin-independent endocytosis. Interestingly, instead of transfer to the lysosome with subsequent degradation, the internalized CT is rapidly sorted into the trans-Golgi network to enter the retrograde trafficking pathway to the ER (Feng et al., 2004). Export of CT-A from the ER into the cytosol was found to be mediated by temperature-sensitive section 61p (Sec61p), a transporter on the ER membrane (Schmitz et al., 2000). Using this unique transport pathway, coadministered antigen appears to be delivered to the cytosol, subsequently leading to the MHC class I-restricted antigen presentation required for the induction of CTL responses (Fraser et al., 2003).

Intriguingly, unlike intact CT, CT-B does not activate immune responses, but instead, it enhances mucosal tolerance induction (Sun et al., 1994). The fact that CT-B does not trigger DC maturation might explain the induction of tolerance rather than adjuvant activity (Lycke, 2004). A different possibility could be the involvement of DCs in the LP because recent evidence has shown that DCs in the LP have the ability to induce tolerance by several different mechanisms, whereas DCs in PPs are capable of activating immune responses (Kelsall and Leon, 2005). Support for this pathway comes from studies demonstrating that CT-B was directly transported to the basolateral side of polarized ECs in the villi (Lencer et al., 1995). However, this issue is still controversial because some reports demonstrated that recombinant CT-B had adjuvant activity in terms of promoting DC maturation and for

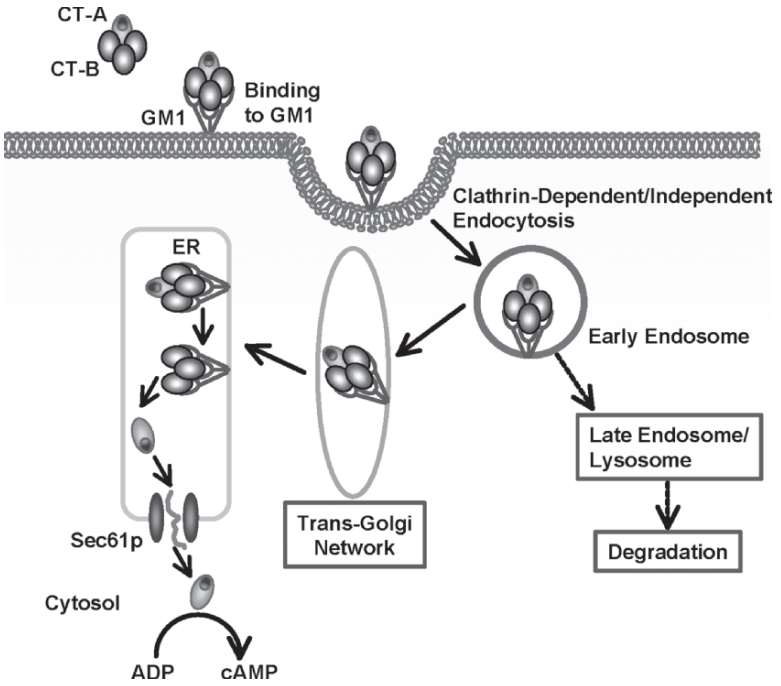

FIG. 14.2. A unique trafficking pathway for CT into the cytoplasm. The intact CT molecule binds to GM1 ganglioside via the CT-B subunit. After endocytosis of CT–GM1, the complex is transported to the endoplasmic reticulum (ER) through a retrograde pathway mediated by the trans-Golgi network. In the ER, CT-A is dissociated from CT-B, and Sec61p mediates the transport of unfolded CT-A into the cytosol, where CT-A becomes folded into the active form with ADP-ribosyltransferase activity.

enhancing mucosal SIgA and systemic IgG Ab responses (Isaka et al., 2004; Isomura et al., 2005).

14.3.2. CT- and LT-Based Mutant Nontoxic Adjuvants

The clinical use of CT and LT has been hampered by the fact that both enterotoxins induce severe diarrhea after oral administration or natural infection. Both enterotoxins also have undesirable side effects involving their entry into the central nervous system when given by the nasal route (Fujihashi et al., 2002). Thus, efforts are now focused on the development of new mucosal adjuvants that do not possess toxicity but that retain adjuvant activity. As described earlier, both CT and LT belong to the AB_5 family of toxins, comprising an A subunit with ADP-ribosylating activity and a pentamer of CT-B subunits that bind to gangliosides on the cell membrane. Thus, several groups including our own have attempted to modify CT-A in order to remove toxicity

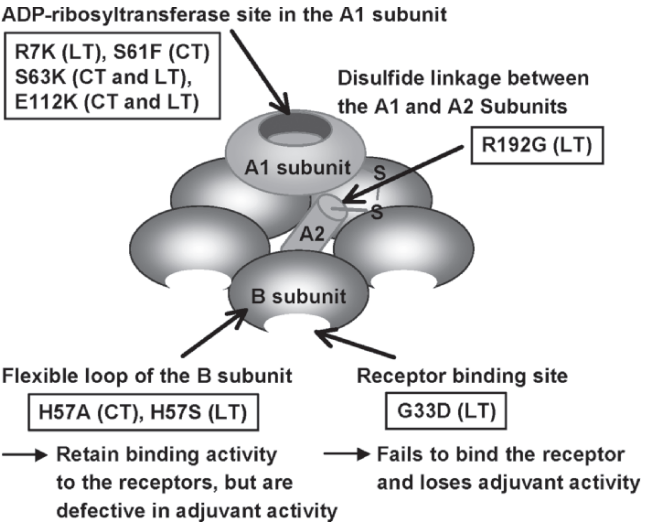

FIG. 14.3. Structure of CT and LT, showing key functional moieties and sites of mutation for generation of mutants of CT and LT as mucosal adjuvants.

(Douce et al., 1995; de Haan et al., 1996; Yamamoto et al., 1997a, 1997b) (Fig. 14.3). In our previous studies, two types of mutants were constructed from CT (Yamamoto et al., 1997a, 1997b). These mutants involved replacement of serine with phenylalanine at position 61 (S61F) or glutamic acid with lysine at position 112 (E112K). Both mutants lacked ADP-ribosyltransferase activity but retained adjuvant activity. Thus, when mutant CTs like native CT were applied nasally, high levels of SIgA Ab and Th2 cell-mediated immune responses were induced.

Subsequently, our studies demonstrated that the mutant forms of CT were effective for the induction of immune responses against tetanus toxin, *Streptococcus pneumoniae*, influenza virus, diphtheria toxin, and botulinum toxin (Kobayashi et al., 2005; Ohmura et al., 2001; Watanabe et al., 2002; Yamamoto et al., 1997a, 1998). Consistent with the numerous successes of mutant forms of CT in the murine system, we have recently reported that the mutant form of CT (E112K) was an effective adjuvant for an anti-human immunodeficiency virus (HIV) vaccine in rhesus macaques, without showing any toxicity (Yoshino et al., 2004). These results offer the possibility of the clinical application of a mutant form of CT (E112K) as a safe and effective mucosal adjuvant. We also constructed a chimeric form of mutant CT containing mutant CT-A (E112K) fused with LT-B. As mentioned earlier, the B subunit of enterotoxin adjuvants determines the direction of Th cell subset that is induced. Consistent with this, the mutant chimera, CT-A(E112K)/LT-B, induced protective immunity in both mucosal and systemic immune compartments against tetanus toxin and influenza virus (Kweon et al., 2002). Most importantly, the immune responses induced by CT-A(E112K)/LT-B

were accompanied by lower IgE Ab responses when compared to mutant CT, suggesting that the chimeric form of mutant CT can induce protective mucosal and systemic immune responses without undesirable allergic responses. A second generation of double mutant CT adjuvants (dmCTs) have recently been constructed, which contain the E112K mutation in the ADP-ribosyltransferase active center as well as mutations in the C-terminal KDEL intracellular targeting motif (Hagiwara et al., 2006). These dmCTs retained strong mucosal adjuvant activity without central nervous system toxicity and may prove to be safer than other mutant forms of CT.

Mutants of LT have also been constructed in attempts to develop a safe mucosal adjuvant (de Haan et al., 1996; Douce et al., 1995; Rappuoli et al., 1995). As with mutant CTs, the main target for mutations has been the LT-A subunit, in order to disrupt ADP-ribosyltransferase activity (Douce et al., 1995). Mutant forms of LT have been demonstrated to enhance protective immunity mediated by SIgA and CTL against measles and influenza viruses, tetanus toxin, *Helicobacter pylori*, and *Streptococcus mutans*, among many others (Barchfeld et al., 1999; De Magistris et al., 1998; Marchetti et al., 1998; Nawar et al., 2007; Partidos et al., 1996). Mutant forms of LT have also shown promise in clinical trials (Peppoloni et al., 2003; Pizza et al., 2000;). Another unique approach has been to detoxify LT by modifying a protease-sensitive residue joining the A1 and A2 subunits (Dickinson and Clements, 1995). The mutant LT (R192G) was used for mucosal adjuvants against infectious diseases like rotavirus, salmonella, *Candida albicans*, and HIV (Cardenas-Freytag et al., 1999; Chong et al., 1998; Morris et al., 2000; O'Neal et al., 1998). The dissociation of the A1 from the A2 subunit is essential for the transport of the A1 subunit from the ER into the cytoplasm, where its ADP-ribosylating function is activated (Fig. 14.2) (Lencer and Tsai, 2003). Thus, the decreased toxicity of LT R192G might be due to the inhibition of LT-A transport into the cytoplasm.

In addition to the A subunit, mutations in B subunits of CT and LT have been examined (Fig. 14.3) (Aman et al., 2001; Fraser et al., 2003; Nashar *et al*., 1996). Disruption of the binding of CT to its GM1-ganglioside receptor resulted in the disruption of adjuvant activity (Nashar et al., 1996). However, a mutant form of CT-B, in which the histidine at position 57 was replaced with alanine, retained the ability to bind GM1-ganglioside receptor but abolished adjuvant activity (Aman et al., 2001). These data indicated that an undetermined activity of CT-B, separate from its ability to bind to surface receptors, is also important in adjuvant activity. Of note, the mutation disrupted the loop structure of the CT-B subunit pentamer. It appears, therefore, that stable conformation of the toxins is important for their adjuvant activity.

14.3.3. Other Toxin-Type Adjuvants

Several other bacterial toxins have been shown to exhibit mucosal adjuvant activity. For instance, Shiga toxin 1 (STX1) and a mutant form of STX1 have recently been shown to exhibit mucosal adjuvanticity. Like CT and LT, STX1

has an AB_5-type structure. Nasal immunization with OVA and a mutant form of STX1 elicited plasma IgG and mucosal SIgA Ab responses (Ohmura-Hoshino et al., 2004). Our recent experiments revealed that mutant STX1 enhanced expression of MHC and costimulatory molecules on DCs (Ohmura et al., 2005). Further, IL-12 and TNF-α expression were enhanced, eventually leading to the effective induction of Th1 and Th2 cell responses as well as the SIgA Ab responses.

Anthrax edema toxin (EdTx), like CT and LT, is an AB_5-type toxin. The A subunit (edema factor) has adenylate cyclase activity, and the B subunits bind to anthrax toxin receptors on target cells. Nasal immunization of mice with OVA antigen plus EdTx induced OVA-specific serum IgG, salivary SIgA, and $CD4^+$ T-cells secreting Th1 and Th2 cytokines (Duverger et al., 2006). An added benefit of this vaccine was the generation of an immune response to the cell-binding B subunit of EdTx. Nontoxic derivatives of EdTx could represent an alternative to ganglioside-binding enterotoxin adjuvants and might be useful for inducing protective immunity against anthrax.

A genetically engineered pertussis toxin (PTX) was developed by removal of its ADP-ribosylating activity and was found to be an effective adjuvant for enhancing mucosal immune responses (Roberts et al., 1995). PTX recognizes N-linked glycan chains containing a branched mannose core and *N*-acetyl glucosamine, found on cell surface glycoproteins of various types of mammalian cell. Nasal immunization with tetanus toxin and PTX augmented parenteral and mucosal Ab responses (Roberts et al., 1995). It should be noted that native PTX has adjuvant activity for enhancement of plasma IgG and mucosal SIgA Ab responses, but it also stimulates production of plasma IgE Abs (Lindsay et al., 1994). However, mutant PTX did not promote IgE responses (Roberts et al., 1995), which alleviated concerns that the adjuvant would induce undesirable allergic responses.

Zonula occludens toxin (Zot) is a single polypeptide encoded by the filamentous bacteriophage infecting toxigenic strains of *V. cholerae* (Baudry et al., 1992). It is capable of disrupting epithelial tight junctions, allowing increased permeability of luminal antigens into mucosal sites via the paracellular route (Fasano et al., 1991). Thus, nasal or rectal immunization with Zot resulted in the induction of plasma IgG and mucosal SIgA Ab responses against coadministered antigens, mediated by both Th1- and Th2-type cells (Marinaro et al., 1999b, 2003).

CTA1-DD is a chimeric adjuvant composed of an enzymatically active CT-A subunit and a dimer of an Ig-binding element of *Staphylococcus aureus* protein A, which targets vaccines to B-cells (Agren et al., 1997). When CTA-DD was applied nasally, it enhanced antigen-specific immune responses in both mucosal and systemic sites without causing inflammation. A subsequent study indicated that the adjuvanticity of CTA1-DD was mediated at least in part by promoting germinal center formation (Agren et al., 2000). Surprisingly, CTA1-DD is nontoxic, although it contains the intact form of CT-A, and both ADP-ribosyltransferase activity and Ig-binding activity are required for

its adjuvanticity (Agren et al., 1999). Furthermore, unlike the mutant form of CT (E112K), a similar mutation in CTA1-DD (CTA1E112K-DD) failed to retain adjuvant activity (Lycke, 2004).

It should be noted that most mutant forms of adjuvants derived from bacterial toxins retained full adjuvant activity after nasal and parenteral immunization but possessed less adjuvant activity when administered orally. The reason for different adjuvant activities after nasal versus oral delivery remains an open question and further experiments are necessary for effective oral delivery use of toxin-based mutant adjuvants.

14.4. Development of Safe and Effective Cytokine- and Chemokine-Based Mucosal Adjuvants

14.4.1. Cytokine-Based Mucosal Adjuvants

Innate-type cytokines have also been successfully used to enhance mucosal immune responses (Table 14.1). As expected from results showing that CT treatment induced IL-1 production, IL-1 itself has been shown to enhance mucosal immune responses to coadministered antigens (Staats and Ennis, 1999).

Table 14.1. Cytokine-, chemokine-, and TLR ligand-based mucosal adjuvants.

Adjuvant	Antibody responses	Th cell responses
Cytokines		
IFN-α/β	Plasma IgG and mucosal SIgA	Th1
IL-1	Plasma IgG and mucosal SIgA	Th2
IL-2	Plasma IgG and mucosal SIgA	Th1 and Th2
IL-12	Plasma IgG and mucosal SIgA	Th1 and Th2
IL-15	Plasma IgG and mucosal SIgA	Th1
IL-18	Plasma IgG and mucosal SIgA	Th1
Chemokines		
Lymphotactin	Plasma IgG, IgE and mucosal SIgA	Th1 and Th2
RANTES	Plasma IgG and mucosal SIgA	Th1
MIP-1α	Plasma IgG only (no mucosal SIgA)	Th1
MIP-1β	Mucosal SIgA with less plasma IgG	Th2
Defensin	Plasma IgG only (no mucosal SIgA)	Th1 and Th2
TLR ligands (receptors)		
MLA (TLR4)	Plasma IgG and mucosal SIgA	Th1 and Th2
MDP (TLR2)	Plasma IgG and mucosal SIgA	Not done
MALP-2 (TLR2/6)	Plasma IgG and mucosal SIgA	Th1 and/or Th2
CpG DNA (TLR9)	Plasma IgG and mucosal SIgA	Th1
Poly (I:C) (TLR3)	Plasma IgG and mucosal SIgA	Th1 and Th2

Abbreviations: MALP, mycoplasma-derived macrophage-activating 2kDa lipopeptide; MDP, muramyl dipeptide; MIP, macrophage inflammatory protein; MLA, monophosphoryl lipid A; RANTES, regulated on activation normal T-cell expressed and secreted; Poly (I:C), polyriboinosinic polyribocytidylic acid; TLR, Toll-like receptor.

Like CT, nasal administration of OVA or tetanus toxoid with IL-1 promoted SIgA and plasma IgG Ab responses with Th2-type helper responses.

Type I interferons (IFNs) (INF-α and IFN-β) were originally identified as antiviral cytokines. Type I IFNs are rapidly produced during bacterial or viral infections and possess multiple pathways to activate the host immune system, including enhancement of cytotoxic activity of natural killer (NK)- and T-cells, upregulation of antigen presentation, and activation of B-cells (Basler and Garcia-Sastre, 2002). Consistent with these activities, it was demonstrated that type I IFN was effective as an adjuvant to enhance systemic IgG, mucosal SIgA, and Th1-type responses (Proietti et al., 2002). Because type I IFNs have already been utilized clinically for the treatment of virus-infected hepatitis patients, they can be considered to be promising candidates for use in humans as mucosal adjuvants.

The well-characterized cytokine IL-2 is mainly produced by CD4-positive ($CD4^+$) T-cells. IL-2 is a lymphoproliferative cytokine and was reported to enhance mucosal and systemic immunity after oral administration (Wierzbicki et al., 2002). This strategy seems to be especially effective in the elderly, because their diminished immune activity is often accompanied by reduced IL-2 production by T-cells (Haynes and Eaton, 2005). In this regard, it was demonstrated that coadministration of IL-2 with papillomavirus pseudoviruses via the oral route restored mucosal and systemic immune responses in aged animals (Fayad et al., 2004). A similar approach might be applied to immunodeficient patients. Recent studies have demonstrated that nasal immunization with a plasmid encoding both IL-2 and antigen is effective for the control of HIV/SIV infection and disease progress in nonhuman primates (Bertley et al., 2004).

The cytokine IL-12 is produced by APCs (e.g., DCs) for induction of Th1-type responses. Like IL-1, IL-12 was found to promote mucosal SIgA and plasma IgG Ab responses when given by the nasal route (Boyaka et al., 1999). The difference between IL-1 and IL-12 was that nasal administration of IL-12 induced both Th1- and Th2-responses (Boyaka et al., 1999). Another approach has involved coadministration of IL-12 with CT. Nasal administration of IL-12 plus CT along with tetanus toxoid antigen preferentially induced Th1-type responses, whereas Th2-type responses were enhanced by oral delivery of CT combined with nasal delivery of IL-12 (Marinaro et al., 1999a). These findings indicate that CT and IL-12 may act through different mechanisms to enhance mucosal immunization, and the determination of Th cell subset induced depends on the route of vaccine delivery. The application of IL-12 as a nasal adjuvant has now been extended to vaccines against numerous infectious diseases, such as tetanus, influenza, the pneumococcus, and *Francisella tularensis* (Arulanandam et al., 1999, 2001; Boyaka et al., 1999; Duckett et al., 2005).

The cytokine IL-15 is produced by DCs, macrophages, and ECs. The IL-15 receptor shares β– and γ-chains with the IL-2 receptor, but it has a unique α-chain that determines its distinct activities (Kovanen and Leonard, 2004).

Both IL-15 and IL-2 activate T- and NK-cells; however, IL-15 preferentially enhances the generation of memory T-cells (Oh et al., 2003). In addition to its effects on T-cells, IL-15 also activates B-cells, particularly B1-cells. IL-15 derived from mucosal ECs was reported to enhance the proliferation and differentiation of B1-cells (but not of B2-cells) into IgA-producing plasma cells in the small intestine (Hiroi et al., 2000). Thus, treatment with IL-15 resulted in the proliferation of B1-cells, whereas anti-IL-15 mAb caused a decrease in the number of B1-cells at the effector sites of the GI and nasal tracts (Hiroi et al., 2000). In accordance with these functions of IL-15, nasal administration of a plasmid encoding IL-15 enhanced Ab and T-cell responses against coexpressed HIV or herpes simplex virus (HSV) antigen for long periods in both mucosal and systemic lymphoid compartments (Toka and Rouse, 2005; Xin et al., 1999).

The cytokine IL-18 has been shown to induce production of IL-12 by DCs and macrophages and thus promotes Th1-type responses. Nasal administration of a plasmid encoding IL-18 plus antigen enhanced SIgA responses in both rectal and vaginal mucosa and subsequently provided protective immunity against HSV-1 (Lee et al., 2003).

Although these cytokines showed adjuvant activities by themselves, studies of the adjuvant activity of cytokines in combination have revealed synergistic effects. For instance, simultaneous administration of IL-1, IL-12, and IL-18 resulted in much stronger adjuvant activity for induction of mucosal SIgA and systemic IgG Abs than that observed with each cytokine alone or with a dual combination of IL-12 plus IL-18 (Bradney et al., 2002). In contrast to the synergistic effects of IL-1, IL-12, and IL-18, co-expression of IL-15 and IL-12 did not enhance adjuvant activity (Xin et al., 1999). These findings suggest that the mechanisms by which cytokines act as immune adjuvants are complex and should be carefully optimized in the development of effective mucosal vaccines against specific infections.

14.4.2. Chemokines and Chemotactic Molecules as Mucosal Adjuvants

Chemokines interact with chemokine receptors on lymphocytes and other cells of the immune system to promote cellular migration along a concentration gradient. Several chemokines were reported to act as innate-type mucosal adjuvants (Table 14.1). For instance, lymphotactin (XCL1) is a C chemokine produced by NK-cells and $CD8^+$ T-cells, including IELs (Boismenu et al., 1996). Lymphotactin promotes chemotaxis of NK-cells and T-cells and has been shown to enhance tumor immunity (Dilloo et al., 1996). When lymphotactin was nasally delivered along with OVA antigen, marked enhancement of OVA-specific SIgA Abs in various mucosal secretions (e.g., feces, saliva, vaginal, and nasal washes) and plasma IgG Ab responses were detected and these responses were supported by both Th1 and Th2 cells (Lillard et al., 1999b).

Mucosal ECs produce RANTES, a CC chemokine, in response to bacterial and viral infections (Saito et al., 1997; Yang et al., 1997). Nasal coadministration of RANTES with OVA induced high levels of SIgA, plasma IgG. and preferential Th1-type responses (Lillard et al., 2001). *In vitro* studies further revealed that expression of CD28, CD40 ligand, and IL-12 receptor on T-cells was increased after treatment with RANTES (Lillard et al., 2001). In spite of the fact that both lymphotactin and RANTES induced mucosal SIgA and plasma IgG Abs, only lymphotactin induced IgE Ab production (Lillard et al., 1999b, 2001). This differential effect might be explained by the different cytokine profiles induced by lymphotactin and RANTES and suggests that, to avoid undesirable allergic responses mediated by IgE, RANTES is likely to be a safer adjuvant than lymphotactin.

Macrophage inflammatory protein (MIP)-1 is another CC chemokine that was analyzed for its ability to act as a mucosal adjuvant. MIP-1 contains two homologous subtypes: MIP-1α and MIP-1β. It was demonstrated that nasal administration of MIP-1α enhanced antigen-specific Ab responses to coadministered antigen in systemic but not in mucosal sites (Lillard et al., 2003). Although MIP-1β shares the same ligand (CCR5), it promoted mucosal SIgA Ab responses with less efficient induction of systemic immune responses (Lillard et al., 2003). These findings indicate that MIP-1α and MIP-1β activate distinct adjuvant pathways, although the mechanisms remain to be elucidated.

Defensins belong to a family of antimicrobial peptides produced by Paneth cells (Selsted and Ouellette, 2005). Defensins also possess chemotactic activity for T-cells and exert adjuvant activity (Lillard et al., 1999a). Coadministration of defensins with antigen promoted Th1- and Th2-mediated systemic IgG Ab responses associated with IFN-γ, IL-5, IL-6, and IL-10 production. It is interesting to note that no mucosal SIgA Ab responses were induced after nasal immunization with defensins. Thus, defensins might be unique adjuvants that enhance systemic immune responses without induction of mucosal SIgA Ab production.

14.5. TLR-Targeted Mucosal Adjuvants

Innate immunity plays a pivotal role in host defense against invading microbial pathogens at early stages of infection. A major breakthrough in our molecular understanding of innate immunity occurred after the discovery of Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns. To date, 13 mammalian TLRs have been identified (Akira and Takeda, 2004). TLR-mediated signals induce cytokine production like type I IFN, IL-1, and IL-12, as well as antimicrobial peptides like defensins, which are all known to have adjuvant activity, as discussed earlier. In addition to the important role of TLRs in innate immunity, accumulating evidence has revealed that TLRs play a pivotal role in the induction of acquired immunity. These attractive features of TLRs might now lead to their application as mucosal adjuvants for the enhancement of SIgA Ab responses (Table 14.1).

14.5.1. Surface TLR-Mediated Adjuvants

Generally, TLR families can be divided into two groups by their cell locale. TLR2 and TLR4 are expressed on the cell surface. The receptor for lipopolysaccharide (LPS) was the TLR to be discovered and is now termed TLR4 (Poltorak et al., 1998). Although LPS itself may be a potent mucosal adjuvant, the medical use of LPS is hampered by its severe toxicity. Thus, recent focus has been aimed at development of an agonist possessing useful immunomodulatory effects without undesirable toxic effects. These efforts have led to the discovery of monophosphoryl lipid A (MLA), a ligand for TLR4, which has already been shown to be a systemic adjuvant preclinically and clinically (Baldridge et al., 2004; Evans et al., 2003). Experimental studies have also demonstrated the effectiveness of MLA as a mucosal adjuvant (Baldridge et al., 2000; Doherty et al., 2002; Pinczewski et al., 2005). Nasal or oral administration of antigens like hepatitis B surface antigen, tetanus toxoid, influenza, *Mycobacterium tuberculosis*, or HIV antigens with MLA promoted SIgA Ab responses in proximal and distal mucosal sites and plasma IgG Abs in systemic sites. The primary target cells of TLR4 agonists are presumably DCs, because TLR4 expression is very low or absent on ECs (Iwasaki and Medzhitov, 2004). In support of this concept, other studies demonstrated that treatment of DCs with MLA induced IL-12 production and enhanced CD80 and CD86 (Martin et al., 2003).

TLR2 is also the target of a specific mucosal adjuvant, muramyl dipeptide (MDP), which is derived from the cell walls of mycobacteria. Prior to its identification as a TLR2 ligands, MDP had been used as an adjuvant for intravaginal and oral immunization (Thapar et al., 1990) and had been shown to stimulate PP cells for the enhancement of IgA Ab responses (Kiyono et al., 1982). Recent studies demonstrated that mycoplasma-derived macrophage-activating 2 kDa lipopeptide (MALP-2) promoted Th2, plasma IgG, and mucosal SIgA responses against coadministered antigens such as β-galactosidase and HIV-1 Tat protein (Borsutzky et al., 2003; Rharbaoui et al., 2002). The adjuvant activity of MALP-2 was associated with binding to heterodimers of TLR2 and TLR6 on NALT B-cells, which induced upregulation of MHC, costimulatory molecules, and CD40 (Borsutzky et al., 2005; Rharbaoui et al., 2004).

14.5.2. Adjuvants Targeting Intracellular TLRs for use with Mucosal Vaccines

The discovery that microbial nucleic acids (e.g., DNA and RNA) have immunostimulatory activity has led to their development as mucosal adjuvants. Bacterial but not eukaryotic DNA generally contains nonmethylated "CpG motifs" and acts as a ligand for TLR9, thus enhancing innate and adaptive immunity (Hemmi et al., 2000). TLR9 is expressed in intracellular compartments of the endosome–lysosome pathway in APCs, and TLR9-mediated

signals have been shown to enhance antigen presentation, expression of costimulatory molecules, including CD80, CD86, and CD40, and production cytokines such as IFN-α/β and IL-12 (Akira and Takeda, 2004). Additionally, CpG directly induces B-cells to proliferate and secrete Ig. In contrast to its intracellular expression on immune cells, it has recently been reported that TLR9 is expressed on the surface of intestinal ECs (Lee et al., 2006). Interestingly, stimulation of TLR9 from the basolateral surface of polarized ECs resulted in NF-kB activation and secretion of IL-8, whereas apical stimulation conferred intracellular tolerance to subsequent TLR challenges. These investigators suggested that the distinctive apical TLR9 signaling in intestinal ECs might contribute to the maintenance of colonic homeostasis in the presence of large numbers of commensal bacteria (see Chapter 10).

TLR9-mediated cellular activation can be achieved by the use of synthetic oligonucleotides (ODN) containing CpG motifs, such as GACGTT in mice (Krieg et al., 1995) and GTCGTT in humans (Hartmann and Krieg, 2000). In addition to the adjuvant effects on systemic immune responses, numerous studies have been focused on the use of CpG ODN as a mucosal adjuvant. Mucosal administration of antigen with CpG ODN has been shown to promote mucosal SIgA, plasma IgG, and T-cell responses, including $CD8^+$ CTLs and $CD4^+$ Th1 cells that stimulate type I IFN production by DCs. The adjuvant activity of CpG ODN has been demonstrated to enhance protective immunity against various types of mucosal infection, such as those caused by *Streptococcus pneumoniae*, HIV, HSV-2, and *Helicobacter pylori* (Chu et al., 2000; Dumais et al., 2002; Gallichan et al., 2001; Harandi and Holmgren, 2004; Horner et al., 2001; Jiang et al., 2003). Comparisons of the adjuvant effects of CpG ODN administered via different mucosal routes demonstrated that nasal and oral delivery resulted in similar enhancement of mucosal and systemic immune responses, whereas the rectal route was less effective (McCluskie and Davis, 2000). Although the clinical use of CpG ODN in mucosal vaccines has not yet been investigated, the numerous successes with CpG ODN as a mucosal adjuvant in experimental animals, as well as several studies with CpG ODN in clinical trials for systemic immunization, should accelerate the acceptance of CpG ODN as a mucosal adjuvant for human vaccines. Finally, from the view of stability, cost, and quality control, CpG ODN seems to be one of the most feasible mucosal adjuvant candidates.

TLR3 and TLR7/8, which act as receptors for double-stranded RNA (dsRNA) and single-stranded RNA, respectively, are, like TLR9, localized in intracellular compartments (Akira and Takeda, 2004). Recent studies demonstrated that nasal delivery of the synthetic dsRNA molecule polyriboinosinic polyribocytidylic acid [poly (I:C)], along with inactivated influenza virus HA, induced protective immune responses against influenza virus infection (Ichinohe et al., 2005). To date, few studies have been performed to analyze the ability of TLR3- and TLR7/8-mediated pathways when given with a mucosal vaccine. However, one can reasonably expect additional studies to examine the feasibility of TLR3- and TLR7/8-targeted mucosal adjuvants

for the induction of protective immunity against various mucosal bacterial and viral infections.

14.6. Mucosal Antigen Delivery Systems

The mode of antigen delivery is another important aspect of mucosal vaccine development. Particulate antigens appear to be more effective than soluble ones, partly due to the protection of the antigen from the harsh conditions of the mucosal environment such as low pH, detergent effects of bile salts, and extensive proteolytic enzyme activity. Additionally, uptake of antigen by M-cells, a key gateway system for antigen sampling, is more effective with particulate antigens (Clark et al., 2001b) (see Chapters 2 and 9). In this section, we will outline various approaches to the development of an ideal mucosal antigen delivery system.

14.6.1. Inert Synthetic and Hybrid Delivery Systems for Mucosal Vaccines

A variety of mucosal antigen delivery systems have been developed using inert particles, including biodegradable polymer-based particles as well as lipid-based particles such as liposomes and ISCOMs (Vajdy et al., 2004). As a representative of polymer-based particles, poly-lactide coglycolide (PLG) microparticles have been extensively investigated (O'Hagan and Singh, 2003). Their biodegradability and easy regulation for controlled drug release have facilitated the application of the PLG system in humans as drug delivery vehicles (Okada and Toguchi, 1995). In the early 1990s, several groups adapted this PLG delivery system to vaccine development and demonstrated that nasal or oral immunization with PLG microparticles encapsulating protein or DNA antigens induced high levels of protective mucosal SIgA and plasma IgG Abs as well as helper T-cells and CTLs (reviewed in O'Hagan and Singh, 2003). Several approaches have been exploited to enhance antigen delivery efficacy by changing the chemical properties of microparticles. For instance, enteric coatings have been employed to protect encapsulated antigens from the acidic gastric environment and to allow the rapid release of antigen in the small intestine (Jain et al., 1996; Vogel et al., 1998). Gelatin capsules have been used for this purpose because they dissolve in the alkaline pH of the intestine but not in the acidic pH of the stomach (Moldoveanu et al., 1993). An additional example would be the chemical mucoadhesive molecules (e.g., carboxy vinyl polymer), which have been used to elongate particles containing protein antigens, thereby prolonging antigen persistence in the intestine (Kunisawa et al., 2000).

Liposomes, spherical particles with a bilayered phospholipid membrane, act as antigen delivery systems for enhancing immune responses by protecting the incorporated antigen from degradation (Somavarapu et al., 2003; Vogel et al., 1998). Liposomes can be prepared with different phospholipid compositions,

thus conferring different chemical and biological properties. Because liposomes are generally unstable in acids, lipases, and bile salts in the GI tract, several efforts have been made to develop more stable forms of liposomes (reviewed in Zho and Neutra, 2002). Previous studies had demonstrated that stable liposomes could be constructed with dipalmitoyl-phosphatidylserine, dipalmitoyl-phosphatidylcholine, and cholesterol (Aramaki et al., 1993; Han et al., 1997). Antigen-specific SIgA responses were induced when mice were immunized with GM1 antigen using these more stable liposomes (Han et al., 1997). Another approach to improve the stability of liposomes in the GI tract was to create the cross-linked network among the lipid membrane by covalent bonds (Chen and Langer, 1997; Okada et al., 1995).

Additional modifications of antigen delivery systems have been attempted in order to target the antigen selectively to M-cells. Some evidence suggests that the physical properties of synthetic particles (e.g., size, hydrophobicity, and surface charge) influence the efficiency of the selective delivery of the encapsulated antigen to M-cells (Clark et al., 2001b). For example, polylactic acid (PLA) microparticles 4 μm in diameter enhanced plasma IgG Abs but not intestinal SIgA Abs, whereas 7-μm PLA microparticles enhanced SIgA Ab production. However, 26-μm microparticles were ineffective because they were too large to be taken up by M-cells in the PP (Tabata et al., 1996).

In addition to physical factors, lectins and microbial adhesins have been widely exploited to enhance access of microencapsulated antigens to M-cells (Jepson et al., 2004). *Ulex europaeus agglutinin* 1 (UEA1), a lectin specific for α-L-fucose residues, has been shown to bind selectively to the apical surface of M-cells of murine PPs and NALT (Giannasca et al., 1994; Takata et al., 2000). Incorporation of UEA1 into microparticles or liposomes resulted in selective and efficient delivery of antigens to M cells after oral administration (Chen et al., 1996; ; Clark et al., 2001a; Foster et al., 1998). The high efficacy of UEA-1-mediated antigen delivery resulted in significant enhancement of OVA-specific Ab responses when mice were immunized orally with microparticles coated with UEA-1 and OVA (Foster and Hirst, 2005). This strategy has now been applied to the induction of mucosal and systemic immune responses to HIV (Manocha et al., 2005; Wang et al., 2005).

Recent advances in biotechnology have resulted in the identification of additional candidate molecules for efficient delivery of antigen to M-cells (Higgins et al., 2004; Lambkin et al., 2003). Organic molecules and peptides that mimic the functional activity of UEA-1 were identified from mixture-based positional scanning synthetic combinatorial libraries and phage peptide libraries, respectively. Synthetic digalloyl D-lysine amide and tetragalloyl D-lLysine amide molecules were found to bind to the surface of M-cells. Coating of microparticles with these compounds resulted in their selective and efficient delivery to M-cells with high efficacy (Lambkin et al., 2003). It was subsequently demonstrated that the synthetic peptide YQCSYTMPHPPV selectively bound to the M-cell-rich subepithelial dome region of the PP and enhanced the delivery of microparticles to M-cells (Higgins et al., 2004).

Another approach has been to apply microbial adhesins for the targeted delivery of synthetic particles to M-cells. Enhanced antigen uptake was achieved by coating polystyrene nanoparticles with *Yersinia*-derived invasin, a ligand for β1 integrins on the apical side of M-cells (Clark et al., 1998). Similarly, reoviruses are known to invade through M-cells using a 45-kDa viral hemagglutinin sigma one (σ1) protein (Forrest and Dermody, 2003). Mucosal immune responses were significantly increased by mucosal immunization of antigen coupled to σ1 protein (Wang et al., 2003; Wu et al., 2001). Combining the advantages of liposomes and specific targeting molecules, we developed a "fusogenic liposome," a hybrid antigen delivery vehicle composed of a synthetic liposome and ultraviolet-inactivated Sendai virus, also known as Hemagglutinating Virus of Japan (Fig. 14.4) (Kunisawa et al., 2001a). Using the fusion activity of Sendai virus, the fusogenic liposomes effectively delivered the encapsulated antigen to NALT ECs, including M-cells, when given nasally (Kunisawa et al., 2001b). Nasal immunization with fusogenic liposomes containing OVA or HIV glycoprotein 160 (gp160) induced high levels of antigen-specific plasma IgG and mucosal SIgA in saliva, fecal extracts, nasal and vaginal washes, as well as CTL responses (Kunisawa et al., 2001b; Sakaue et al., 2003). Similar viruslike particles (e.g., influenza virus) have been developed for targeted delivery of protein antigens administered by the nasal route (Lambkin et al., 2004).

Immune stimulating complexes (ISCOMs) are another category of lipid-based vehicles that has been well studied as a mucosal delivery system. ISCOMs consist of a particle 30–40 nm in diameter composed of phospholipid, cholesterol, and Quil A or QS-21 (the purified component from Quil A). Quil A is a saponin adjuvant originally isolated from *Quillaja saponaria* (Kensil et al., 1991). The saponin intercalates with the cell membrane of APCs to form pores, thus delivering antigen into the cytoplasm where presentation by the MHC class I pathway leads to induction of CTLs (Bangham et al., 1962; Sanders et al., 2005). QS-21 has also been shown to enhance production of the Th1-type cytokines IFN-γ and IL-12 (Mikloska et al., 2000; Silla et al., 1999). ISCOMs initially were applied to parental vaccination for prevention of infectious diseases caused by influenza virus and HIV-1 (Rimmelzwaan et al., 1997; Takahashi et al., 1990). The first application of ISCOMs for mucosal vaccine delivery was a study of oral immunization with OVA antigen (Mowat et al., 1991). Oral delivery of ISCOMs containing OVA induced antigen-specific mucosal SIgA, plasma IgG, Th1/Th2, and CTL responses. Subsequent studies demonstrated the effectiveness of ISCOMs for enhancing protective immune responses against viral infections such as influenza, rotavirus, and HSV-2 (Fooks, 2000; Kazanji et al., 1994; Mohamedi et al., 2000; Simms et al., 2000; van Pinxteren et al., 1999). As a possible mechanism for the mucosal adjuvant activity of ISCOMs, one study has demonstrated that oral feeding of ISCOMs resulted in the recruitment and activation of DCs in the mesenteric lymph nodes and PPs (Furrie et al., 2002). Similarly, nasal immunization using ISCOMs was quite effective for inducing mucosal SIgA, plasma IgG, and CTL responses for protective immunity against influenza

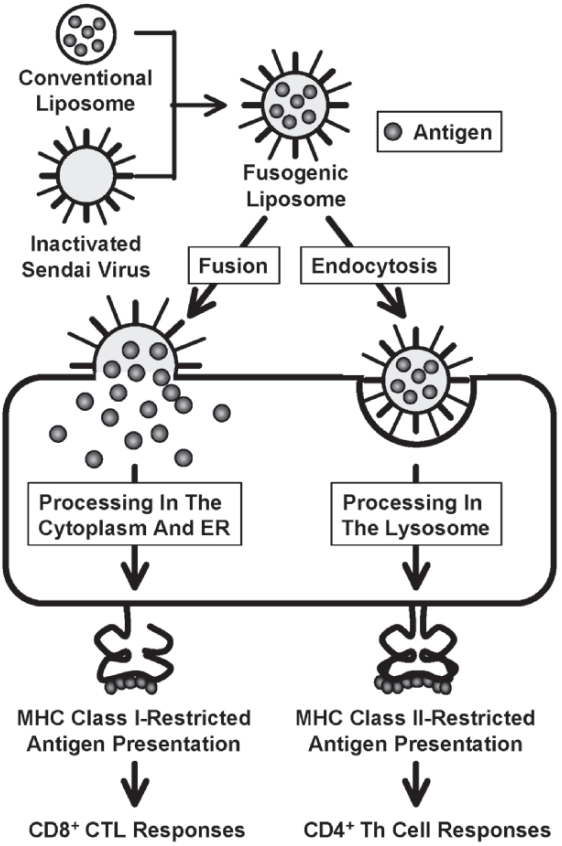

FIG. 14.4. A hybrid delivery vehicle, termed fusogenic liposome, used for the induction of CTL and SIgA Ab production via MHC class I- and II-restricted antigen presentation, respectively. Fusogenic liposomes were prepared by fusing conventional liposomes with ultraviolet-inactivated Sendai virus. Fusogenic liposomes can deliver the encapsulated antigen into the cytoplasm via its fusion with the cell membrane. The endocytosis pathway also participates in the uptake of the fusogenic liposome. The dual pathways for fusogenic liposome uptake allow antigen presentation restricted by both MHC class I and II molecules, leading to $CD8^+$ CTL and $CD4^+$ Th cell responses, respectively.

virus, HSV-2, and respiratory syncytial virus infections (Ben-Ahmeida et al., 1994; Hu et al., 1998, 2001; Ugozzoli et al., 1998).

14.6.2. Genetically Modified Live Microorganisms for Antigen Delivery

Historically, inactivated vaccines have been prepared from microorganisms inactivated by formaldehyde or β-propiolactone treatment. However, these inactivated forms of vaccine do not induce protective mucosal immune

responses unless they are coadministered with mucosal adjuvants. On the other hand, live attenuated microorganisms have been shown to be highly effective as mucosal vaccines (e.g., poliovirus, *Salmonella typhi* Ty21a, and *Vibrio cholerae*) (Dietrich et al., 2003). Unlike chemical inactivation, the attenuation process may not affect the activity of naturally expressed ligands for M-cells, which promote vaccine uptake via M-cells for the effective induction of mucosal and systemic immune responses. Therefore, live attenuated microorganisms are taken up by M-cells into the PPs or NALT, which results in the efficient priming of antigen-specific immune responses.

The effective uptake of live attenuated microorganisms by M-cells has led to the development of recombinant attenuated microorganisms to deliver heterologous antigen. Recent progress in recombinant DNA technology has allowed the creation of a new generation of mucosal vaccines, in which live attenuated microorganisms are engineered to carry DNA encoding heterologous antigen (Curtiss, 2005). To ensure both safety and effectiveness, several genes determining pathogenicity are mutated or disrupted, and a gene encoding a heterologous antigen is inserted. An important consideration in the development of recombinant live attenuated vaccines for viral, parasitic, and fungal pathogens is that the vaccine antigen should be expressed in the host eukaryotic cells, as appropriate glycosylation and folding are required for immunogenicity of the expressed protein antigens (Darji et al., 1997). This can be accomplished through the use of intracellular bacteria as vaccine vectors, which invade host cells and release plasmid DNA encoding the vaccine antigen. On the other hand, for protection against bacterial infections, it is necessary that the antigens be expressed within the recombinant bacteria that are used as the vaccine vector.

Several types of recombinant attenuated bacteria have been considered as candidates for delivery vehicles of DNA encoding heterologous vaccine antigens, which can be divided into invasive and noninvasive types. The former include attenuated strains of *Salmonella typhi*, *Shigella flexneri*, and *Listeria monocytogenes,* and the latter include *Vibrio cholerae*, *Lactobacllus* spp., and *Yersinia enterocolitica* (Curtiss, 2005). Important attributes of candidate bacterial vaccine vectors include the ability to survive in the hostile environment of the GI and respiratory tracts and to bind to M-cells in order to selectively enable the effective induction of mucosal and systemic immune responses (e.g., mucosal SIgA, plasma IgG, and CTL). It is also critical that the attenuated bacterium does not revert back to a virulent form capable of triggering disease symptoms.

Among the bacteria described earlier, *Salmonella* are the best studied in regard to attenuating mutations. The licensed oral *S. typhi* vaccine strain, Ty21a, was prepared by nitrosoguanidine mutagenesis. In addition to nonspecific chemical mutagenesis, attenuation by targeted gene disruption has been investigated since the early 1970s. The *gal*E gene was the first target for site-directed mutagenesis (Germanier and Furer, 1971). This mutation decreased UDP-galactose epimerase activity but was not successful for vaccine purposes because the mutant bacteria were not immunogenic. In contrast, *Salmonella* strains with a deletion in the *aro* genes, which blocks the biosynthesis of the aromatic amino acids

tyrosine, phenylalanine, and tryptophan, were both avirulent and immunogenic (Dougan *et al*., 1987; Hoiseth and Stocker, 1981). However, administration of high doses of *S. typhimurium* strains with *aro* mutations to humans caused potentially dangerous bacteremia (Hone et al., 1992). Therefore, an additional attenuating mutation in the *htr*A gene was performed (Tacket et al., 1997b). Deletion of the *htr*A gene, which encodes a heat stress protein, results in a less virulent organism because of reduced survival and replicative ability (Johnson et al., 1991). The doubly mutated *Salmonella* strain successfully induced antigen-specific Ab production and T-cell responses in humans when given by the oral route, without causing undesirable side effects (Tacket et al., 1997b). Additional gene deletions, including those encoding adenylate cyclase, the cyclic AMP receptor protein (*cya*), and the regulatory system for phosphate sensing (*pho*), were also tested for vaccine development against *Salmonella* (Curtiss and Kelly, 1987; Hohmann et al., 1996; Tacket et al., 1997a). Similar approaches have been employed to generate mutants of other bacteria, such as *Shigella*, *L. monocytogenes,* and *Mycobacterium bovis* (also known as Bacille Calmette-Guerin, BCG) for vaccine use (Dramsi et al., 1996; Haile and Kallenius, 2005; Levine et al., 1997).

Using these mutant forms, recombinant bacteria have been engineered to express heterologous antigen by plasmid transformation. It should be noted that amplification of the transformed plasmids and expression of antigenic protein must be tightly regulated, as overexpression of antigens may cause the death of the bacteria due to a metabolic burden (Galen and Levine, 2001). One possible solution to this problem is the use of specific promoters that are active only after entry into host cells. The most extensively examined promoters include the anaerobically inducible *nir*B promoter, the temperature-inducible promoter *htr*A, and the macrophage-inducible promoter *pag*C (Chatfield et al., 1992; Everest et al., 1995; Hohmann et al., 1995). These delivery systems have proven effective for induction of antigen-specific SIgA Ab and CTL responses against various virulence determinants of pathogens like diphtheria exotoxin, HIV, HBV, and pertussis (Barry et al., 1996; Gomez-Duarte et al., 1995; Grillot-Courvalin et al., 1998; Hone et al., 2002; Karem et al., 1997; Woo et al., 2001; Wu et al., 1997).

Similarly, recombinant viruses have been developed as vehicles for mucosal vaccine delivery. Efforts have been aimed at developing mucosal vaccines that induce both mucosal SIgA Abs to prevent initial contact of pathogens with host cells in mucosal sites and antigen-specific CTLs for surveillance of virus-infected cells. Several types of virus have the advantage of their natural tropism for mucosal sites, including DNA viruses such as poxvirus, adenovirus, HSV, and adeno-associated virus, and RNA viruses such as alphaviruses (e.g., Semliki Forest virus and Sindbis virus), vesicular stomatitis virus, and poliovirus (Ertl and Xiang, 1996; Khromykh, 2000).

The poxviruses, including smallpox, vaccinia, and the avian poxvirus, comprise the best studied DNA viruses for vaccine vectors. Poxviruses have a double-stranded DNA genome ranging in size between 130 and 300 kb. The gene

encoding heterologous antigen is usually inserted at nonessential sites, such as the viral thymidine kinase locus (Mackett et al., 1984). It was determined that modified or synthetic promoters were required to achieve sufficient expression of heterologous antigen, because the endogenous poxvirus promoters (P7.5 and H5) were too weak (Ourmanov et al., 2000; Wyatt et al., 1996). Modified vaccinia virus Ankara (MVA) is the best candidate in the poxvirus family for vaccines due to their relative safety. MVA is an attenuated strain generated by extensive passage in chicken embryo fibroblasts (Stickl et al., 1974). Another safe strain is NYVAC, which was generated by the deletion of 18 genes encoding virulence factors (Tartaglia et al., 1992). Both MVA and NYVAC expressing heterologous antigen elicited strong mucosal SIgA and plasma IgG Abs as well as CTL responses when they were administered via mucosal routes, leading to their testing as potential vectors for an HIV vaccine (Drexler et al., 2004; Hutchings et al., 2005; Paoletti, 1996; Sauter et al., 2005).

Adenovirus is another DNA virus that has been investigated for mucosal vaccine development and for gene therapy. Adenoviruses have several advantages over other viruses, as they can be readily obtained in high titers and stored at room temperature after freeze-drying. Most importantly, adenovirus naturally infect via mucosal routes; thus, they can deliver the antigen into mucosal sites with great efficacy. Adenoviruses are nonenveloped and contain a linear double-stranded DNA genome between 30 and 45 kb in size. Replication-defective adenovirus vectors have been constructed by insertion of genes encoding heterologous antigens into the E1 region, which is essential for viral replication (Mizuguchi et al., 2001). The E3 and E4 regions of the adenovirus genome have also been found suitable for cloning of heterologous genes (Mizuguchi et al., 2001). These recombinant adenoviruses have been shown to elicit potent Ab and cellular immune responses against antigens when they were applied by mucosal routes (e.g., oral, nasal, rectal, and vaginal), resulting in protective immunity against viral infections, including HBV, HSV, influenza virus, and HIV (Babiuk and Tikoo, 2000; Santosuosso et al., 2005). The success of experimental adenovirus-mediated mucosal vaccines against HIV has led to their use in clinical trials (Barouch and Nabel, 2005; Gomez-Roman and Robert-Guroff, 2003).

A novel adenovirus-derived delivery system has recently been described for mucosal targeting of a vaccine against botulinum neurotoxin A (BoNT/A) (Maddaloni *et al.*, 2006). A chimeric protein was constructed in which the cell-binding domain in the C terminus (Hc) of BoNT/A was fused to adenovirus 2 fiber protein (Ad2F), which binds to receptors on respiratory epithelial cells. Nasal immunization of mice with Hc-Ad2F plus CT adjuvant induced robust intestinal SIgA and plasma IgA responses, which were protective against subsequent lethal BoNT/A challenge.

Alphaviruses have received attention as a mucosal antigen delivery system due to their high efficiency of gene transduction and apparent safety (Lundstrom, 2002). In contrast to the DNA viruses like poxvirus and adenovirus, alphaviruses have an RNA genome and replicate in the host cytosol, which

provides safety advantages by eliminating the possibility of integration of heterologous antigen into the host genome. In this family, Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus have been well studied for expression of heterologous antigen (Karlsson and Liljestrom, 2003; Lundstrom, 2003b). These viruses contain a single-stranded positive-sense RNA genome, 11–12 kb in size, which consists of two open reading frames (ORFs). The heterologous antigen is generally inserted into the ORF encoding the viral structural protein. This cloning strategy results in the generation of mutant viruses that are incapable of producing viral progeny. Significant mucosal SIgA, plasma IgG, and CTL responses have been observed in response to mucosal administration of recombinant alphaviruses, which induced protective immunity against a broad range of viral, bacterial, and parasitic pathogens (Lundstrom, 2003a).

Poliovirus also belongs to a family of positive-stranded RNA viruses. Since attenuated poliovirus has been used clinically for many years, its safety is widely accepted. Polioviruses are resistant to the harsh conditions in the GI tract and they bind to M-cells after oral inoculation (Sicinski et al., 1990). Both replication-competent and replication-defective polioviruses have been developed as heterologous antigen delivery vehicles. Replication-competent polioviruses were engineered with a modified capsid protein expressing heterologous antigen (Andino et al., 1994). In the replication-defective poliovirus, the structural proteins (VP2 and VP3) were replaced with genes encoding heterologous antigen (Porter et al., 1993). Both systems were shown to be effective as mucosal antigen delivery systems and generated protective immunity against infectious diseases such as HIV (Crotty and Andino, 2004).

It should be noted that both bacteria-based and virus-based vaccines are not only effective for the delivery of antigens but also possess natural adjuvant activities because they express ligands for TLR family members. A potential concern, however, is that antivector immune responses may develop after repeated immunization, hampering the reuse of the recombinant vaccine for boosting of the immune response.

14.7. Concluding Remarks

Infectious diseases remain a threat to human health in both developing and advanced nations. It is crucial to develop effective mucosal vaccines against pathogens that induce both mucosal and systemic immune responses, especially SIgA and plasma IgG Abs as the first and second layers of humoral protection. In addition, the induction of mucosal CTLs is needed for protective immunity against viral pathogens. Recent advances in biomedical engineering and progress in understanding the cellular and molecular immunology of infectious diseases have allowed the development of versatile mucosal vaccine systems, based on novel mucosal adjuvants and mucosal antigen delivery systems. Creation of a new generation of mucosal vaccines

requires coordination of multiple factors, including the choice of the antigen delivery system and the optimal mucosal adjuvant. In this regard, it might be necessary to compensate weak points of one with strong attributes of the other, by exploiting potential synergistic effects among different adjuvants and delivery systems. Although further investigation is clearly needed, there is no doubt that novel mucosal adjuvants and antigen delivery systems will facilitate the development of a new generation of mucosal vaccines that will contribute to human health.

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