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Mucosal Vaccines: An Overview

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Chapter 47

The exposure of mucosal tissues to antigens (Ags) results in the induction of local and disseminated responses in the mucosal compartment as well as systemic immune responses manifested by the presence of Ag-specific antibodies (Abs) in external secretions and plasma, and T cells of various subsets in both mucosal and systemic lymphoid tissues. Thus, both mucosal and systemic immune compartments respond, under normal conditions, to a vast spectrum of environmental antigens of mainly food and microbial origin. As described in preceding chapters, this enormous antigenic load has resulted in a strategic distribution of cells involved in the uptake, processing, and presentation of Ags, the production of Abs, and cell-mediated immune (CMI) T-cell defenses at the front line of mucosal tissues.

For almost 100 years, researchers have attempted to design vaccines to be administered by a variety of mucosal routes rather than by conventional parenteral injections (see the Historical Introduction at the beginning of this book). Mucosal vaccination has many attractive features, including easy and painless administration, potential for mass immunization in case of emergencies, and reduced cost of production, storage, and delivery. More important, only mucosal vaccines consistently promote immune responses at the most common sites of entry of infectious agents. These desirable features immediately prompt a question: why do we have so few mucosal vaccines? As will become obvious from the ensuing chapters in this section, the optimal doses of Ag for

mucosal vaccination are difficult to establish because of the low and unpredictable absorption from intestinal surfaces and the interference with quantitatively superior antigens in the gastrointestinal (GI) tract. Therefore, a need to develop methodologies that would mediate the preferential absorption of desired Ags is obvious. Furthermore, because of the presence of proteolytic enzymes in external secretions, most Ags need to be protected from digestion (see Chapter 1). To enhance the magnitude or quality of immune responses, many mucosal adjuvants have been extensively tested in experimental animals and to a very limited degree in humans (see Chapter 54). Although many of these substances displayed desired effects, their acceptance in humans is restricted because of their potential toxicity (*e.g.*, cholera toxin [CT] and the heat-labile toxin [LT] of *E. coli*) and low adjuvanticity in humans as opposed to animals (*e.g.*, QS-21); however, some mucosal adjuvants have not been adequately evaluated in humans.

Although the stimulation of protective immune responses to mucosal infectious agents is the ultimate criterion for a vaccine's performance, the possibility of induction of a state of systemic unresponsiveness to mucosally administered antigen—mucosal tolerance—has been frequently considered to be a negative factor in acceptance of mucosal vaccines. Mucosal tolerance is indeed a fundamental feature of the mucosal immune system and a critical functional component that efficiently prevents and suppresses otherwise

unavoidable overstimulation of the entire immune system by environmental Ags. Thus, the enhancement of protective mucosal immune responses to infectious agents that is sought by vaccinologists and the suppression of systemic responses may seem paradoxical. As discussed next, such outcomes are not mutually exclusive because of a hierarchy in the quality of immune responses.

INDUCTIVE AND EFFECTOR SITES AND THE COMMON MUCOSAL IMMUNE SYSTEM

Extensive studies concerning the origin of B- and T-lymphocytes that ultimately populate mucosal tissues and secretory glands and of immunization routes effective in the induction of mucosal immune responses indicated that the common mucosal immune system (CMIS) can be divided into two functionally distinct compartments, namely, inductive versus effector sites. This network is highly integrated and finely regulated, and the outcome of mucosal tissue encounters with foreign Ags and pathogens can range from mucosal and plasma Abs, T-cell CMI, and cytotoxic T-lymphocyte (CTL) responses, on the one hand, to systemic anergy or mucosal tolerance on the other. This physiological division is of paramount importance in the design of vaccines effective for the induction of protective immunity within the mucosal immune system and, in particular, its humoral branch. Experiments performed in animal models revealed that the inductive sites present in certain locations, such as gut-associated or in some species bronchus-associated lymphoepithelial tissues (GALT, represented by Peyer's patches, and BALT, respectively), function as primary sources of precursor cells which migrate through the lymphatics and blood and after directed extravasation populate remote mucosal tissues and glands (Phillips-Quagliata and Lamm, 1988; Scicchitano *et al.*, 1988). More recent studies suggest that such inductive sites are not necessarily restricted to Peyer's patches found mainly in the small intestine and the BALT in bronchi. Additional sites have been identified in nasal mucosa; palatine tonsils and other organized lymphoid tissues of Waldeyer's ring in the nasopharynx (Kuper *et al.*, 1992; Kiyono, 1997); the large intestine, especially the rectum; and the genital tract.

Numbers and types of cells involved in immune responses and their products, primarily Abs and mediators (cytokines, chemokines), are remarkably different in the mucosal and systemic compartments of the immune system. Thus, secretory IgA (S-IgA) differs from plasma IgA not only in terms of specific Ab activity but also in the proportions of polymeric versus monomeric forms and of origin in secretory tissues versus bone marrow plasma cells. The ontogenies of the mucosal and systemic IgA compartments display characteristic and apparently independent patterns of maturation. Adult levels of IgA are reached in external secretions considerably earlier (1 month to 2 years) than in the blood plasma (adolescence) (Allansmith *et al.*, 1968; Mellander *et al.*,

1984). Experiments addressing the origin of mucosal Abs have led to the clear conclusion that an overwhelming proportion of such Abs are produced locally in mucosal tissues and that only a minor fraction derive from the circulation in most species, including humans (Brandtzaeg, 1984; Mestecky and McGhee, 1987; Mestecky *et al.*, 1997).

Inductive sites of the gastrointestinal tract

Inductive sites for mucosal immune responses were initially described in mucosal locations such as small intestinal Peyer's patches of the GALT (Phillips-Quagliata and Lamm, 1988). Earlier studies have shown that surgical removal of Peyer's patches from the intestine of rats does not affect the total number of IgA-containing cells in the lamina propria or the development of a normal antibody response to intestinal immunization (Heatley *et al.*, 1981). More recently, mice lacking Peyer's patches by *in utero* treatment with a lymphotoxin- β receptor-Ig molecule fusion protein developed normal mucosal and systemic immunity to oral immunization (Yamamoto *et al.*, 2000) suggesting that alternative inductive sites for mucosal immunity are present in the GALT. In fact, other lymphoid follicles, termed "isolated lymphoid follicles (ILFs)" or "solitary lymphoid follicles," were reported in the intestinal wall of rabbits (Keren *et al.*, 1978), guinea pigs (Rosner and Keren, 1984), and humans (Moghaddami *et al.*, 1998; Neutra *et al.*, 2001). These structures have now been demonstrated in mice (Hamada *et al.*, 2002; Fagarasan *et al.*, 2003; Lorenz *et al.*, 2003), where their number and maturation appear to be triggered by luminal stimuli such as the bacterial flora (Fagarasan *et al.*, 2003; Lorenz *et al.*, 2003).

Pharyngeal and nasal lymphoepithelial tissues

Organized lymphoid tissues, including palatine, lingual, and nasopharyngeal tonsils (Waldeyer's ring), are strategically positioned at the beginning of the digestive and respiratory tracts and are continuously exposed to ingested and inhaled antigens. These nasopharyngeal-associated lymphoepithelial tissues (NALT) possess structural features similar to both lymph nodes and Peyer's patches of the GALT (Brandtzaeg, 1984; Ogra, 1971; see Chapter 83). For example, tonsillar crypts possess a lymphoepithelium which contains M cells for selective antigen uptake as well as B and T cells, plasma cells, and antigen-presenting cells (APCs). The distribution of IgA1- and IgA2-producing cells in the nasal and gastric mucosa and in lacrymal and salivary glands is similar to the distribution in tonsils, suggesting that tonsils may serve as a source for precursors of IgA plasma cells found in the upper respiratory and digestive tracts. Furthermore, tonsillectomized children display lower levels of S-IgA antibodies to the oral poliovirus vaccine than children with intact tonsils (Ogra, 1971). Others have shown that direct unilateral injection of antigens (cholera toxin B subunit [CT-B] and tetanus toxoid [TT]) into the tonsil of human volunteers resulted in the induction of mainly mucosal immune responses, manifested by the appearance of antigen-specific IgG and, to a lesser degree, IgA-forming cells (AFCs) in the injected ton-

sil (Quiding-Järbrink *et al.*, 1995a). Recent studies confirm the prevalence of IgG antibodies in tonsils and demonstrated that these structures display features of both mucosal inductive and effector sites (Boyaka *et al.*, 2000). Therefore, the tonsils may serve as an inductive site analogous to GALT in some species. Organized BALT were also noted at airway branches of experimental animals, although these structures rarely occur in humans (Pabst, 1992).

Several studies have emphasized the importance of inductive sites in the nasal cavity for the generation of mucosal and systemic immune responses that may exceed in magnitude those induced by oral immunization (Bergquist *et al.*, 1997; Di Tommaso *et al.*, 1996; Gallichan and Rosenthal, 1995; Lubeck *et al.*, 1994; Pal *et al.*, 1996; Russell *et al.*, 1996; Staats *et al.*, 1996). When introduced into the nasal cavity, usually along with mucosal adjuvants such as CT and/or CT-B, viral and bacterial antigens induce superior immune responses in external secretions such as saliva and, surprisingly, in female genital tract secretions of rodents, rhesus monkeys, chimpanzees, and humans (Quiding-Järbrink *et al.*, 1995b; Russell and Mestecky, 2002). Whether such antibody responses are also induced in the male genital tract remains to be determined. Nevertheless, this finding may have important implications for the design of vaccines effective in the induction of immune responses in the genital tract (Russell and Mestecky, 2002; see Chapter 95). Although analogous studies with bacterial antigens have not been performed in humans, induction of genital tract immune responses by nasal immunization would have profound implications for the prevention of sexually transmitted diseases, including AIDS (see Chapter 52). Thus, different mucosal immunization routes (nasal and oral) can induce generalized mucosal immune responses, although the relative representation of dominant antibody isotypes may vary. Circulating IgA AFCs induced after nasal vaccination express a more promiscuous profile of homing receptors than their corresponding counterparts raised after oral or rectal immunization (Quiding-Järbrink *et al.*, 1997; Kantele *et al.*, 1998). This could explain the fact that nasal immunization appears to induce S-IgA immunity in a broader range of mucosal tissues than oral vaccination.

Lymphoepithelial tissues in the large intestine and rectal immunization

Although most investigations of GALT mucosal IgA inductive sites have primarily centered on Peyer's patches and the appendix, analogous follicular structures are also found in the large intestine, with especially pronounced accumulations in the rectum (Langman and Rowland, 1986; O'Leary and Sweeney, 1986). The potential importance of rectal lymphoid tissues as an IgA inductive site and as a source of IgA plasma cell precursors is suggested by several studies. The predominance of IgA2 cells over IgA1 cells in the lamina propria of the large intestine clearly diverges from the relative apportioning of the IgA subclass distribution in other mucosal tissues (Crago *et al.*, 1984; Kett *et al.*, 1986). That this is also the case in the female genital mucosal tissues

(uterus, cervix, fallopian tubes, and vagina) (Kutteh *et al.*, 1988) suggests that rectal lymphoid tissues may be an important source of IgA precursors destined for the genital tract. The potential importance of rectal lymphoid tissues as an inductive site is suggested from several studies performed with humans, nonhuman primates (NHPs), and mice. Rectal immunization of humans with a microbial vaccine (*e.g.*, *Salmonella typhi* Ty21a) or various viruses induced specific antibodies not only at the site of immunization but also in saliva and other secretions (Forrest *et al.*, 1990; Kozlowski *et al.*, 1997, 2002; Russell and Mestecky, 2002; see Chapters 94 and 95). The rectal route of immunization has also been evaluated in animal experimentation. Rhesus macaques immunized rectally with simian immunodeficiency virus (SIV) displayed both T- and B-cell-mediated immune responses, including the induction of anti-SIV antibodies (Lehner *et al.*, 1992, 1993). Mice immunized rectally with CT or recombinant vaccinia virus expressing gp120 of SIV also generated humoral immune responses in genital tract secretions as well as in serum; this immunization route was frequently superior to either the gastric or vaginal route (Moldoveanu *et al.*, 1995; Haneberg *et al.*, 1994). Therefore, the rectal immunization route appears to be effective in the induction of not only local but also generalized mucosal immune responses because of the presence of inductive site tissue. However, there are pronounced species differences with respect to the magnitude of the immune response induced: mice display a more vigorous response than humans. The type and dose of antigen, as well as the frequency of immunization, may be partially responsible for such observed differences. Further studies will be necessary to validate limited results obtained thus far, and males will be included in future immunization attempts to determine whether specific immune responses are generated in male genital tract secretions.

MUCOSAL HOMING AND COMPARTMENTALIZATION OF MUCOSAL IMMUNE RESPONSES

Early evidence of selective mucosal homing was revealed by the finding that rabbit Peyer's patch B cells repopulated the gut and became IgA plasma cells (Craig and Cebra, 1971, 1975). Further, the mesenteric lymph nodes of orally immunized experimental animals were found to contain antigen-specific precursors of IgA plasma cells, which repopulated the lamina propria of the gut and mammary, lacrimal, and salivary glands (McDermott and Bienenstock, 1979; McWilliams *et al.*, 1975, 1977; Roux *et al.*, 1977). Other evidence of the existence of the CMIS in humans was provided by the finding of specific S-IgA antibodies in secretions of the intestinal, respiratory, and genital tracts, as well as in tears, saliva, and milk, and by the observation of IgA-secreting cells in peripheral blood following oral immunization (Czerkinsky *et al.*, 1987; Kantele, 1990; Mestecky, 1987; McGhee *et al.*, 1992). Of importance for vaccine

development, as mentioned earlier, is that immunization at certain inductive sites can give rise to a humoral immune response preferentially manifested at certain effector sites and resulting in further subcompartmentalization of the CMIS. For example, repeated nasal immunization may induce elevated mucosal immune responses not only in nasal secretions but also in saliva. On the other hand, rectal vaccination may induce preferential immune responses at the site of immunization and in some instances in the female genital tract.

A number of studies have now established that the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is the major homing receptor ligand in the GALT (Berlin *et al.*, 1993; Holzmann *et al.*, 1989; Bell and Issekutz, 1993; Hamann *et al.*, 1994; Rott *et al.*, 1996; see Chapter 34). In addition, pairing of $\alpha 4$ with $\beta 7$ represents the major integrin molecule responsible for lymphocyte binding to MAdCAM-1 expressed on high endothelial venules (HEVs) in Peyer's patches (Holzmann *et al.*, 1989). In contrast to the GALT, L-selectin and peripheral lymph node addressins play predominant roles in the binding of naive lymphocytes to NALT HEVs (Csencsits *et al.*, 1999, 2001). Additional studies showed expression of L-selectin by most effector B cells induced by systemic immunization, with only a small proportion expressing $\alpha 4\beta 7$, while the opposite was seen after enteric (oral or rectal) immunization (Quiding-Jabrink *et al.*, 1995b, 1997). Interestingly, effector B cells induced by nasal immunization displayed a more promiscuous pattern of adhesion molecules, with a large majority of these cells expressing both L-selectin and $\alpha 4\beta 7$ (Quiding-Jabrink *et al.*, 1997).

It is now clear that chemokines are directly involved in mucosal homing of effector B and T cells. For example, loss of secondary lymphoid tissue chemokine (SLC) results in lack of naive T-cell or dendritic cell (DC) migration into spleen or Peyer's patches (Gunn *et al.*, 1999). Further, thymus-expressed chemokine (TECK) mediated human memory T cell migration into the small intestinal lamina propria of the GI tract. In fact, gut homing $\alpha 4\beta 7^{\text{hi}}$ T-cell expression of the TECK receptor CCR-9 (Zabel *et al.*, 1999) and reduction in levels of total intraepithelial lymphocytes (IELs) occur in the small intestine of CCR9^{-/-} mice (Wurbel *et al.*, 2001). In addition to its chemoattractant activity on T cells, TECK efficiently recruits IgA-producing but not IgG- or IgM-producing cells from spleen, Peyer's patches, and the mesenteric lymph nodes (Bowman *et al.*, 2002). While all CD4⁺ and CD8⁺ T lymphocytes in the small intestine express high levels of CCR9, only a small subset of lymphocytes in the colon are CCR9⁺ (Kunkel *et al.*, 2000). Lymphocytes from other tissues, including tonsils, lung, inflamed liver, normal or inflamed skin, inflamed synovium and synovial fluid, breast milk, and seminal fluid, do not express CCR9 (Kunkel *et al.*, 2000). Consistent with CCR9 expression, TECK expression is also restricted to the small intestine (Kunkel *et al.*, 2000). These findings imply a restricted role for lymphocyte CCR9 and its ligand TECK in the small intestine and add support to the functional compartmentalization of

immune responses in different segments of the gastrointestinal (GI) tract and the CMIS in general.

Recent studies have shown that CCR10, a receptor for the mucosa-associated epithelial chemokine MEC (CCL28), is selectively expressed by IgA⁺CD38^{hi}CD19^{int/}CD20⁻ IgA antibody-secreting cells, including circulating IgA⁺ plasmablasts and almost all IgA⁺ plasma cells in the salivary gland, the small and large intestines, the appendix, and the tonsils (Kunkel *et al.*, 2003; Lazarus *et al.*, 2003). Further, MEC attracts IgA-producing but not IgG- or IgM-producing AFCs from the intestines, lungs, and lymph nodes draining the bronchopulmonary tree and oral cavity (Lazarus *et al.*, 2003). Interestingly, T cells from mucosal sites fail to respond to MEC, suggesting that distinct chemokine signaling is needed for mucosal homing of B versus T cells. This concept is further supported by the fact that CCR9, whose ligand TECK/CCL25 is predominantly restricted to the small intestine and thymus, is expressed by a fraction of IgA antibody-AFCs and almost all T cells in the small intestine but by only a small percentage of plasma cells in other sites (Kunkel *et al.*, 2003; Lazarus *et al.*, 2003).

ROUTES OF IMMUNIZATION FOR PROTECTION OF MUCOSAL SURFACES

Mucosal immunization

Mucosal routes remain the preferred ones for vaccine delivery for induction of mucosal immunity. However, application of antigens to mucosal areas that lack inductive sites such as intestinal Peyer's patches is usually inefficient in the generation of disseminated mucosal and systemic immune responses. For example, the administration of nonreplicating antigens in the conjunctival sac, vagina, or intestinal loops, without Peyer's patches, results in a local, low immune response (for review see Mestecky, 1987; Mestecky and McGhee, 1987). Similarly, injection of antigens into the lactating mammary or salivary glands induces S-IgA and high IgG antibody responses in milk and saliva collected from immunized glands but not in secretions of other mucosal sites (for review see Mestecky, 1987). A concomitant IgG response in plasma suggests that injection of antigens into effector sites should be considered as systemic rather than mucosal immunization. This also seems to be the case when antigens are injected in the vicinity of regional lymph nodes draining certain mucosal sites.

Systemic immunization

Systemic immunization generally elicits weak humoral responses in external secretions and cellular responses in mucosal tissues. However, external secretions of the female and male genital tracts (cervical mucus and vaginal wash, and pre-ejaculate and ejaculate) contain approximately equal levels of IgA and IgG of mostly circulatory origin (see Chapter 96). Therefore, systemic immunization may be of considerable importance in the induction of protective responses in the genital tract secretions. Furthermore, systemic immunization

with conjugated polysaccharide (*Haemophilus influenzae* or *Streptococcus pneumoniae*)–protein tetanus toxoid (TT) or diphtheria toxoid (DT) vaccine induce both IgA and IgG responses in plasma and secretions and predominantly IgA-secreting cells in the peripheral blood of systemically immunized volunteers (Lue *et al.*, 1990; Fattom *et al.*, 1990). Until the recent approval of the cold-adapted live attenuated influenza virus nasal vaccine (FluMist; MedImmune Vaccines, Inc., Gaithersburg, MD) in June 2003, systemic immunization with inactivated or split influenza virus has been and may continue to be the vaccine of choice. Thus, systemic immunization can be used as an effective route of antigen administration and ensuing protection in several mucosally contracted diseases (see Chapter 91).

Another important aspect of systemic immunization concerns its primary effects on subsequent mucosal immunization. Many previous studies indicate that single and even repeated immunization at the same mucosal site is not a particularly effective mode of induction of vigorous immune responses. Instead, empirical experience convincingly demonstrates that a combination of mucosal immunization routes (*e.g.*, oral and rectal) elicits better responses. Of utmost importance for induction of responses to HIV-1 is the need for the stimulation of both mucosal and systemic immunity. This goal may not be easily attainable by strictly mucosal (*e.g.*, oral or nasal) immunization. However, the combination of systemic priming and mucosal boosting will most likely lead to the desired outcome. Previous experiments (Moldoveanu *et al.*, 1993) have shown that systemic priming, even with minute doses of antigens (in this case the influenza virus) followed by mucosal boosting, elicited better mucosal and systemic immune responses than did mucosal immunization only. Furthermore, systemic priming does not preclude humoral and CMI responses induced by mucosal boosting (Belyakov *et al.*, 1999)—a distinct advantage of this sequence of immunization. The reversed order of immunizations—mucosal priming and systemic boosting—may create some undesirable complications (Czerkinsky *et al.*, 1999). In a recent phase II clinical trial, intramuscular immunization with a live recombinant canarypox HIV-1 vaccine could induce CTL responses in both the systemic and mucosal compartments (Musey *et al.*, 2003). The study also showed that CD8⁺ CTL clones established from rectal and systemic cells of one vaccine recipient exhibited similar Env-specific responses and major histocompatibility complex (MHC) restriction (Musey *et al.*, 2003). This result suggests that parenteral vaccination can induce HIV-1-specific CTLs that localize to sites of HIV-1 infection.

Transdermal immunization

Immune responses have been induced through transdermal (skin) immunization by scarification such as in smallpox inoculation (Benenson *et al.*, 1977). More recently, the skin emerged as an attractive target for vaccine delivery. For example, depilatory agents (Tang *et al.*, 1997) and epidermal powder delivery system were shown to promote both mucosal and systemic immunity (Chen *et al.*, 2001). More interestingly, several studies in humans (Glenn *et al.*, 2000) and animal models

(Glenn *et al.*, 1998; Sharton-Kerten *et al.*, 2000; Beignon *et al.*, 2001; Anjuere *et al.*, 2003; Guebre-Xabier *et al.*, 2003) suggest that, in addition to systemic immunity, mucosal immunity can be achieved by topical application to intact skin of a vaccine antigen and the appropriate adjuvant. Adjuvants including CT, CT-B, LT, nontoxic mutants of LT, CpG, and cytokines promoted immunity to cutaneous vaccines (Sharton-Kersten *et al.*, 2000; Beignon *et al.*, 2001; Chen *et al.*, 2001; Anjuere *et al.*, 2003), suggesting that enhancement of the immune response to topical vaccines is not restricted to native enterotoxins. Epidermal dendritic cells (DCs) or Langerhans cells appear to play a major role in the immune responses to transcutaneous immunization (Baca-Estrada *et al.*, 2002). External factors such as vitamin D or ultraviolet radiation B were also reported to regulate systemic and mucosal immunity to transcutaneous vaccines (Enioutina *et al.*, 2002). However, the mechanisms underlying the induction of mucosal immunity by transcutaneous vaccines remain elusive.

PROTECTIVE MECHANISMS INDUCED BY MUCOSAL VACCINES

S-IgA antibodies

Most if not all current vaccines exert their protective effect through the induction of specific Abs. Mucosal Abs, which are predominantly IgA and to a lesser degree IgG, exhibit their protective function by mechanisms that are distinct from specific antibodies present in the plasma (see Chapter 14). Inhibition of microbial adherence is a critical initial step for the protection of the host and is mediated by both specific and nonspecific mechanisms. For instance, the agglutinating ability of S-IgA specific to capsular polysaccharide of *H. influenzae* seems to be crucial for preventing colonization by *H. influenzae* (Kauppi-Korkeila *et al.*, 1996). Furthermore, another nonspecific mechanism that inhibits microbial adherence is due to the presence of carbohydrate chains on the S-IgA molecule that bind to bacteria or other antigens (Davin *et al.*, 1991; Wold *et al.*, 1990). S-IgA antibodies have been shown to be effective in neutralizing viruses at different steps in the infectious process. In particular, S-IgA specific to influenza hemagglutinin can interfere with the initial binding of influenza virus to target cells or with the internalization and the intracellular replication of the virus (Armstrong and Dimmock, 1992). The S-IgA can neutralize the catalytic activity of many enzymes of microbial origin (such as neuraminidase, hyaluronidase, glycosyltransferase, and IgA-specific protease) as well as the toxic activity of bacterial enterotoxins (CT and the related LT of *E. coli*). *In vitro* experiments employing murine polarized epithelial cells have demonstrated that antibodies specific for rotavirus and hepatitis virus can neutralize the respective viruses inside the epithelial cells (Mazanec *et al.*, 1992, 1995), and evidence has been provided that similar mechanisms occur *in vivo* (Burns *et al.*, 1996). Similarly, it has been shown that transcytosis of primary HIV isolates is blocked by polymeric IgA specific to HIV envelope proteins (Bomsel *et al.*, 1998). It should be mentioned that S-IgA antibodies appear to be

important in limiting inflammatory responses at mucosal surfaces. In fact, IgA antibodies are unable to activate complement and interfere with IgM- and IgG-mediated complement activation (Griffiss and Goroff, 1983; Russell and Mansa, 1989). Furthermore, IgA can downregulate the synthesis of TNF- α and IL-6 as well as enhance the production of IL-1R antagonists by LPS-activated human monocytes (Wolf *et al.*, 1994, 1996). An additional anti-inflammatory mechanism of S-IgA-mediated protection has been recently suggested. Thus, S-IgA can colocalize with LPS in the apical recycling endosome compartment, preventing LPS-induced NF- κ B translocation and a subsequent proinflammatory response (Fernandez *et al.*, 2003).

Mucosal CTLs

There is a clear demarcation between inductive sites, which harbor precursor CTLs (pCTLs), and effector sites, which include lamina propria and the epithelial cells where activated CD8⁺ CTLs function. It is now established that administration of virus into the GI tract results in a higher frequency of pCTL in Peyer's patches (Offit *et al.*, 1991; London *et al.*, 1987). For example, reovirus localizes to T-cell regions and is clearly associated with increased CD8⁺ pCTLs and memory B cell responses (London *et al.*, 1990). Oral administration of *Vaccinia* to rats resulted in the induction of virus-specific CTLs in Peyer's patches and mesenteric lymph nodes (Issekutz, 1984). Virus-specific CTLs are also generated in mucosa-associated tissues by oral immunization with reovirus and rotavirus (Offit *et al.*, 1991; London *et al.*, 1987), and a high frequency of virus-specific CTLs are present in the Peyer's patches as early as 6 days after oral immunization. These studies suggest that oral immunization with live virus can induce antigen-specific CTLs in both mucosal inductive and effector tissues for mucosal responses and in systemic lymphoid tissues as well.

The vaginal and gastrointestinal models of NHP infection with SIV have been useful to demonstrate that successful vaccine protection against SIV/HIV may require CTL responses in the mucosa. It has been shown that vaginal inoculation of NHPs with a low dose of pathogenic SIV induces CTLs, which could develop in the absence of detectable antibodies (McChesney *et al.*, 1998) and protect or delay the onset of disease upon challenge (Wilson *et al.*, 2000). Other studies have shown CTL responses in GI mucosal tissues of chronically SIVmac-infected NHPs, which contained levels of CTLs comparable to those found in the peripheral blood and lymph nodes (Schmitz *et al.*, 2001). Finally, while high CTL responses develop at a similar rate and magnitude in both peripheral and mucosal lymphoid tissues in primary SIV infection, mucosal CTL responses may predominate later in the course of the disease (Veazey *et al.*, 2003).

Cytokines/chemokines in mucosal immunity

The role of T helper (Th) cell-derived cytokines for antibody and CMI responses is now well established. Thus, interleukin (IL)-12, IL-18, and IFN- γ trigger Th0 cells to differentiate along the Th1 pathway (Kobayashi *et al.*, 1989; Chan *et al.*, 1991; Micaleef *et al.*, 1996; Okamura *et al.*, 1998). Mature

Th1 cells produce IL-2, IFN- γ , and lymphotoxin- α (LT- α , also known as TNF- β), LT- β , and TNF- α (Mosmann and Coffman, 1989) and mediate Th1-type responses that are associated with CMI and the IgG2a antibody subclass in the mouse system (Snapper and Paul, 1987). On the other hand, IL-4 promotes differentiation of Th2 cells that produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Mosmann and Coffman, 1989) and provide effective help for IgG1, IgG2b, and IgE antibody responses (Coffman *et al.*, 1988; Finkelman *et al.*, 1990; Esser and Radbruch, 1990). Both Th1 and Th2 cells are quite sensitive to cross-regulation. Thus, IFN- γ inhibits both Th2 cell proliferation and B-cell isotype switching stimulated by IL-4 (Gajewski and Fitch, 1988). Likewise, IL-10 inhibits IFN- γ secretion by Th1 cells, allowing the development of Th2-type cells. Further, the Th2 cell subset is an effective helper phenotype for supporting the IgA isotype in addition to IgG1, IgG2b, and IgE responses in the mouse system.

Earlier studies established a major role for IL-5 and IL-6, two Th2-type cytokines, for inducing sIgA⁺ B cells to differentiate into IgA-producing plasma cells in both mice (Beagley *et al.*, 1988, 1989, 1991) and humans (Fujihashi *et al.*, 1991). Thus, IL-10 has also been shown to play an important role in the induction of IgA synthesis, especially in humans (Briere *et al.*, 1994; Defrance *et al.*, 1992). Finally, high frequencies of Th2 cells producing IL-5, IL-6, and IL-10 were shown in mucosal effector sites (*e.g.*, the intestinal lamina propria and the salivary glands) where IgA responses occur (Mega *et al.*, 1992; Taguchi *et al.*, 1990). More recent studies with a number of mucosal adjuvants and vaccine delivery systems have now shown that Th1-type cytokines are also important for mucosal S-IgA (Van Cott *et al.*, 1996; Marinaro *et al.*, 1997; Arulanandam *et al.*, 1999; Boyaka *et al.*, 1999). This finding underlines the importance of S-IgA for protection of host mucosal surfaces against both soluble toxins and allergens as well as intracellular pathogens that require complement-fixing antibodies and CMI.

It is now clear that Th1- and Th2-type cells express distinct patterns of chemokine receptors (Bonnacchi *et al.*, 1998; Sallusto *et al.*, 1998; Kim *et al.*, 2003). For example, CCR5 and the CXC chemokine receptors CxCR3 and CxCR5 are preferentially expressed by human Th1 cell clones, while Th2 cells express CCR4 and to a lesser extent CCR3 (Sallusto *et al.*, 1998; Imai *et al.*, 1999). It has also been shown that mouse Th1 cells but not Th2 cells express CCR7 (Randolph *et al.*, 1999). Interestingly, only CCR7-⁺ Th2 cells localize in the periphery of the T-cell zones, from where they can provide help to nearby B cells in B-cell follicles (Randolph *et al.*, 1999). To what extent such dichotomy between localization of Th1 and Th2 cells occurs in mucosal inductive and effector tissues remains to be determined.

MUCOSAL IMMUNE RESPONSES TO VACCINES

Almost all viral and bacterial pathogens to which vaccines would be desirable, invade mucosal tissues where cell-medi-

ated and antibody-mediated immunity would be most effective. It is therefore striking that very few of the current vaccines were given by a mucosal route until the recent approval of the nasal FluMist influenza vaccine (**Table 47.1**). As indicated earlier in this chapter, mucosal delivery of vaccines has to overcome a number of problems, including vaccine stability and optimal antigen dosing in mucosal inductive sites. The most effective mucosal adjuvants are enterotoxins (*i.e.*, CT and LT) and, as such, are unsuitable for use in humans (see Chapter 54). Until recently, their mechanisms of action were poorly understood, and this has hampered the development of safe mucosal vaccines. However, the interest in mucosal vaccine development has increased significantly in the scientific community and has led to over 2,000 publications on adjuvants and delivery systems for mucosal vaccines during the last 5 years (**Figure 47.1**). Studies over the past decade have now identified the sites of actions and several mechanisms that contribute to the mucosal adjuvanticity. Thus, mucosal adjuvants can act through pattern recognition such as Toll-like receptors (TLRs) on epithelial cells (**Figure 47.2**). They can also improve antigen delivery in mucosal inductive sites by increasing the permeability of the epithelial layer or by targeting M cells (**Figure 47.2**). More recently, studies have established that APCs are key players in the development of mucosal immunity. In fact, adjuvants including enterotoxins and cytokines upregulate costimulatory and MHC class II molecule expression by macrophages (M ϕ) and DCs and thus favor their interaction with effector B and T cells (Cong *et al.*, 1997; Yamamoto *et al.*, 1999; Gagliardi *et al.*, 2000; Staats *et al.*, 2001) (**Figure 47.2**). In addition, cytokines secreted by APCs, including IL-1, IL-12, and IL-18, act as adjuvants for induction of antibody and CMI responses (Staats *et al.*, 2001; Bradney *et al.*, 2003). Cytokines such as Flt3 ligand (Flt3 L) or GM-CSF, which increase the number of DCs and stimulate their maturation (Williamson *et al.*, 1999; Dauer *et al.*, 2003), also promote

immunity to mucosal vaccines. Mucosal adjuvants can stimulate maturation and differentiation of effector B cells and trigger B-cell switching for production of S-IgA antibodies (**Figure 47.2**). They may also influence the development of targeted Th1-type responses for protection against intracellular pathogens or Th2-type responses required for protection against soluble antigens, allergens, and toxins (**Figure 47.2**). As described below, CT and LT virtually employ all the mechanisms listed earlier to enhance mucosal immunity, confirming these enterotoxins as the preferred model systems for studying regulation of mucosal immunity by mucosal adjuvants (**Table 47.2**).

Enterotoxins and nontoxic derivatives as mucosal adjuvants

The major enterotoxins produced by *Vibrio cholerae* and *Escherichia coli*, CT and LT, respectively, have continued to be the most studied mucosal adjuvants (see Chapter 54) and account for nearly 40% of all the publications on mucosal vaccines during the last 5 years (**Figure 47.1**). The B subunits of these molecules bind to cell surface gangliosides and thus could target vaccines to mucosal epithelial cells. CT and LT could also enhance immune responses by increasing the permeability of epithelial membranes. Studies in the 1980s and early 1990s showed the role of CT for B-cell switching and S-IgA antibody production. It has also become clear over the past few years that mucosal adjuvants play a major role in the differentiation of CD4⁺ T cells and subsequent Th1- or Th2-type responses for protection against intracellular pathogens or toxins. In this regard, the mucosal adjuvant activity of CT after oral immunization of mice with protein antigen and CT as adjuvant involves IL-4 and Th2-type responses (Xu-Amano *et al.*, 1993; Marinaro *et al.*, 1995; Vajdy *et al.*, 1995). On the other hand, oral immunization with LT resulted in mixed CD4⁺ Th1- and Th2-type responses that included IFN- γ , IL-5, IL-6, and IL-10

Table 47.1. Mucosal Vaccines for Humans

Vaccine	Route	Comments
Polio vaccine of Sabin	Oral	Used to eradicate polio (no longer used in the United States)
Flu-Mist Trivalent: cold-adapted live influenza vaccine (MedImmune Vaccines, Inc)	Nasal	Approved in the United States since June 2003
<i>Salmonella typhi</i> Ty21A: Live typhoid vaccine (Vivotif Berna vaccine, Berna Biotech ag)	Oral	Licensed in the United States (not available in the United States)
CT-B / Killed whole-cell cholera vaccine (Dukoral, Chiron)	Oral	Approved in 18 countries including Canada; awaiting approval in Europe
CVD 103-HgR: Single-dose cholera vaccine (Orochol or Mutacol, Berna Biotech ag)	Oral	Pending registration in the United States
Nonliving nasal influenza HA plus <i>E. coli</i> LT adjuvant (Berna Biotech ag)	Nasal	Off market; associated with cases of Bell's palsy
Rhesus reassortant rotavirus vaccine (Rotashield, Wyeth-Lederle Vaccine)	Oral	Withdrawn from the market in 2000; cases of intussusception

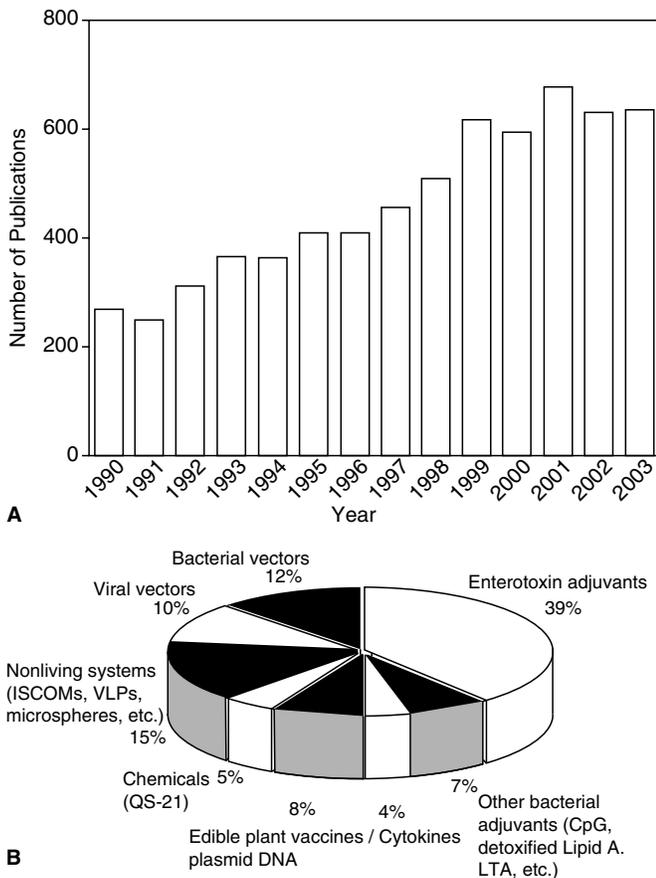


Fig. 47.1. **A**, Numbers of publications since 1990 on the subject of mucosal vaccine or mucosal immunization, taken from MEDLINE and additional life science journals (PubMed, U.S. National Library of Medicine). By mid November in 2003 alone, 561 manuscripts had been published. **B**, The percentage of publications for each major category of mucosal adjuvants and delivery systems, since 1999.

production (Takahashi *et al.*, 1996). Studies with chimeras of CT and LT have shown that the nature of the subunits of these enterotoxins, but not the ADP ribosyltransferase activity, direct the type of CD4⁺ T-cell responses (Bowman and Clements, 2001; Kweon *et al.*, 2002; Boyaka *et al.*, 2003). It has now been established that enterotoxins upregulate costimulatory and MHC class II molecule expression by M ϕ and DCs and thus favor their interaction with effector B and T cells (Cong *et al.*, 1997; Yamamoto *et al.*, 1999; Gagliardi *et al.*, 2000). Further, CT induces IL-1 secretion by APCs, and this cytokine has now been shown to promote mucosal immunity by mechanisms resembling those mediated by CT (Staats *et al.*, 1999). The mutants of CT and LT (mCTs and mLTs) generated by amino acid substitution are generally poor adjuvants when given by the oral route (see Chapter 54). Since safety issues are associated with the use of enterotoxins as nasal adjuvants (see next section), the next important challenge for mucosal vaccinologists will be to devise

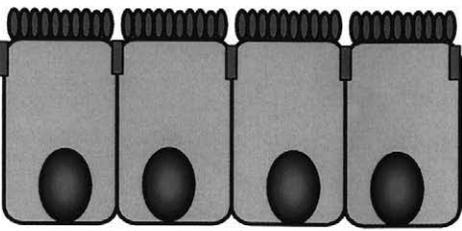
enzymatically inactive mCTs and mLTs capable of acting as mucosal adjuvant for oral vaccines.

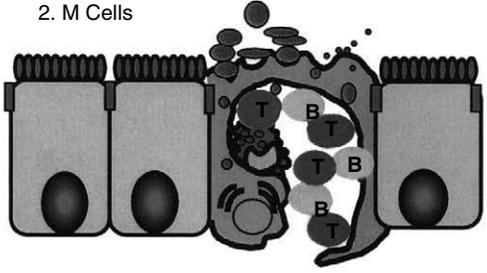
Immunostimulatory DNA sequences

It is now clear that stimulation via pattern recognition such as TLRs favor the development of mucosal immunity. Bacterial but not eukaryotic DNA contain immunostimulatory sequences consisting of short palindromic nucleotides centered around a CpG dinucleotide core, *e.g.*, 5'-purine-purine-CG-pyrimidine-pyrimidine-3' or CpG motifs (Krieg, 2002; see Chapter 53). It is now clear that CpG motifs can induce B-cell proliferation and Ig synthesis as well as secretion of cytokines (*i.e.*, IL-6, IFN- α , IFN- β , IFN- γ , IL-12, and IL-18) by a variety of immune cells (Tighe *et al.*, 1998). Since CpG motifs create a cytokine microenvironment favoring Th1-type responses, they can be used as adjuvants to stimulate antigen-specific Th1-type responses or to redirect harmful allergic or Th2-dominated autoimmune responses. Indeed, coinjection of bacterial DNA or CpG motifs with a DNA vaccine or with a protein antigen promotes Th1-type responses even in mice with a pre-existing Th2-type of immunity (Roman *et al.*, 1997; Klinman *et al.*, 1996). More important for this chapter, CpG motifs can enhance systemic as well as mucosal immune responses when given to mice by the nasal route (McCluskie and Davis, 1998; Moldoveanu *et al.*, 1998; Gallichan *et al.*, 2001) or oral route (McCluskie *et al.*, 2000). A recent study provided evidence that CpG oligodeoxynucleotides (ODNs) could also induce innate immune protection of the female genital tract (Harandi *et al.*, 2003). In fact, vaginal administration of CpG ODNs rapidly induced IFN- γ , IL-12, IL-18, and RANTES production in the genital tract mucosa (Harandi *et al.*, 2003). The vaginal CpG ODN treatment protected mice against vaginal challenge with otherwise lethal doses of herpes simplex virus type 2, demonstrating the induction of innate immunity (Harandi *et al.*, 2003). It is interesting that two of the innate molecules induced by CpG (*i.e.*, IL-12 and RANTES) were reported to bridge the mucosal innate with the adaptive immune system (see next section).

Zonula occludens toxin

Zonula occludens toxin (Zot) is produced by toxigenic strains of *Vibrio cholerae* and has the ability to reversibly alter intestinal epithelial tight junctions, allowing the passage of macromolecules through the mucosal barrier. Nasal immunization of mice with a protein antigen and recombinant Zot, either alone or fused to the maltose-binding protein (MBP-Zot), induced high antigen-specific IgA antibody titers in plasma, as well as in vaginal and intestinal secretions (Marinaro *et al.*, 1999a). Moreover, Zot as adjuvant induced antigen-specific IgG subclasses that consisted of IgG1, IgG2a, and IgG2b antibodies and resembled the pattern induced by LT (Marinaro *et al.*, 1999). Zot was recently shown to also act as adjuvant for rectal immunization (Marinaro *et al.*, 2003). These studies illustrate the importance of increasing the permeability of mucosal tissues for induction of mucosal immunity to vaccines.

	Mechanisms	Enterotoxins	Others
<p>1. Epithelium</p> 	<p>Pattern recognition / toll-like receptors</p> <p>Permeability</p> <p>MHC Class II</p>	<p>Cholera toxin, LPS, CpG</p> <p>Cholera toxin, <i>Zonula occludens</i>, LT</p> <p>Cholera toxin, <i>Salmonella</i> LT?</p>	<p>LTA, etc</p> <p>Toxin, cytokines</p> <p><i>shigella</i>, etc.</p>

<p>2. M Cells</p> 	<p>Targeting / uptake</p>	<p>Cholera toxin, Sigma (σ1) reovirus LT?</p>	<p>CT-B / microspheres? ISCOMs?</p>

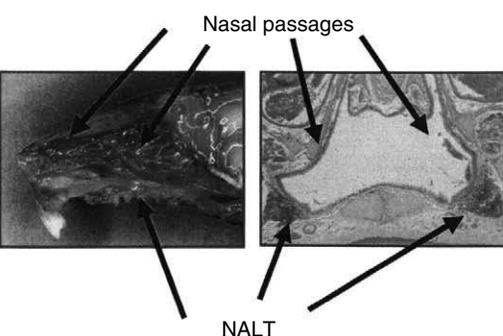
<p>3. GALT / NALT</p> <p>Peyer's patches (GALT)</p>  	<p>APCs (DCs/Mϕ) functions B7-1 / B7-2 MHC Class II</p> <p>DC - CD4⁺ T cell interactions B7 / MHC II CD40</p> <p>CD4⁺ T cell differentiation Th2 Th1</p> <p>Th1 / Th2 - B cell interactions IgG subclass IgE / IgA Abs</p> <p>B-Cell switching Mucosal S-IgA</p>	<p>Cholera toxin, LT?</p> <p>Cholera toxin, LT?</p> <p>Cholera toxin LT</p> <p>Cholera toxin, LT</p> <p>Cholera toxin, LT</p>	<p>Cytokines / chemokines</p> <p>Flt3 L</p> <p>IL-4, IL-13 CpG, IL-12, Vectors</p> <p>Many mucosal adjuvants</p> <p>Many mucosal adjuvants</p>

Fig. 47.2. Sites and mechanisms of action of mucosal adjuvants for induction of mucosal antibodies and CMI responses. Mucosal adjuvants promote mucosal immunity by a variety of mechanisms, including stimulation of epithelial cells, targeting/enhancing Ag uptake, activation of mucosal APCs, and stimulation of effector B and T cells.

Cytokines and chemokines as adjuvants for mucosal Abs and CMI

The use of cytokines and chemokines to enhance immune responses to mucosal vaccines is an attractive strategy for at least two reasons. First, cytokines and chemokines act by often known mechanisms through specific interactions with corresponding receptors. Further, mucosal delivery of cytokines could help avoid the toxicity that is often associ-

ated with the large and repeated parenteral cytokine doses generally needed (Marinaro *et al.*, 1997, 1999b; Huber *et al.*, 2003). Finally, cytokines/chemokines that influence the development of Th cell subsets can help promote targeted Th1-type responses for protection against intracellular pathogens or Th2-type responses required for protection against soluble antigens, allergens, and toxins.

Table 47.2. Mucosal Adjuvants in Common Experimental Use

Bacterial Products	Viral Products	Others
Native enterotoxins CT, LT CT-B, LT-B CT-A, LT-A	Reovirus protein sigma one ($p\sigma 1$)	Cytokines IL-1, IL-12, GM-CSF, Flt3 L
Enterotoxin derivatives Mutant CTs Mutants LTs Chimera of CT-LT		Chemokines RANTES, Lymphotactin, MIP-1 β
LPS and derivatives Zonula occludens toxin CpG ODNs		Saponins (QS-21)

Cytokines as mucosal adjuvants

Consistent with the fact that CT induces IL-1 secretion by APCs and epithelial cells (Bromander *et al.*, 1991, 1993), IL-1 has been shown to enhance mucosal immune responses to coadministered antigens and to promote a pattern of plasma IgG subclasses similar to the Th2-type response inducer CT (Staats *et al.*, 1999). Both CMI and CTL responses could also be induced when IL-1 was nasally administered in combination with the Th1-type inducing cytokines IL-12, IL-18, or IL-12 plus GM-CSF (Bradney *et al.*, 2002). CT has also been shown to induce IL-6 secretion by epithelial cells (McGee *et al.*, 1993). However, in contrast to IL-1, nasal delivery of IL-6 together with a protein antigen promoted antigen-specific systemic immunity but failed to induce mucosal S-IgA antibody responses (Boyaka *et al.*, 1999). Thus, not all cytokines induced by CT can provide signals for mucosal S-IgA antibody responses. IL-12 is a major cytokine produced by APCs in response to a variety of stimuli. This cytokine has been shown to induce antibodies to nasally coadministered protein vaccines in both the systemic compartment and mucosal tissues (Aralandandam *et al.*, 1999; Boyaka *et al.*, 1999) via mechanisms involving CD4⁺ Th1 cells (Boyaka *et al.*, 1999). In addition, nasal (Marinaro *et al.*, 1999b; Belyakov *et al.*, 2000) or oral (Marinaro *et al.*, 1999b) coadministration of IL-12 and CT redirected CT-induced Th2-type responses toward a Th1-type and optimized the induction of CTLs by a mucosal HIV peptide vaccine (Belyakov *et al.*, 2000). A single intratracheal dose of Flt3 L was recently reported to increase the number of DCs and T cells in rat lung tissues and to enhance mucosal antibody responses to a protein antigen delivered thereafter (Pabst *et al.*, 2003). Along the same line, nasal coadministration of a protein antigen and a plasmid DNA expressing Flt3 L (pFlt3 L) promoted antigen-specific mucosal and systemic antibody responses (Kataoka *et al.*, 2004). Interestingly, unlike CpG ODNs, which stimulate Th1-type responses, pFlt3 L promoted Th2-type responses (Kataoka *et al.*, 2004).

Induction of mucosal immunity by chemokines as adjuvant

Innate molecules secreted in mucosal epithelia have also been tested to determine their potential to provide signals to bridge the innate with the adaptive mucosal immune systems. Lymphotactin (Lptn) is a C chemokine produced predominantly by NK and CD8⁺ T cells, including $\gamma\delta$ TCR⁺ IELs. Nasal coadministration of Lptn and a protein antigen enhanced antigen-specific antibody responses both in blood plasma and in mucosal secretions (Lillard *et al.*, 1999). Lptn as adjuvant induced antigen-specific CD4⁺ Th1- and Th2-type cells and IgG1>IgG2a=IgG2b=IgG3 antibody subclasses (Lillard *et al.*, 1999). Another CC chemokine, RANTES, also displayed mucosal adjuvant activity for nasally coadministered protein antigen (Lillard *et al.*, 2001). RANTES as adjuvant promoted antigen-specific CD4⁺ Th1-type cytokine responses and supported Th1-associated plasma IgG subclass responses (Lillard *et al.*, 2001). As was discussed for CT and LT above, similar chemokines may have different effects on mucosal antibody and CMI responses. For example, MIP-1 α and MIP-1 β are distinct but highly homologous CC chemokines that share affinity for CCR5. MIP-1 α also binds CCR1 as well as CCR3 in the mouse, while MIP-1 β is a ligand for CCR8. A recent study has shown that nasally delivered MIP-1 α promotes strong plasma IgG antibodies as well as mucosal and systemic CMI responses to coadministered antigen (Lillard *et al.*, 2003). On the other hand, MIP-1 β was a less effective adjuvant for plasma IgG antibodies and failed to induce CMI responses. However, MIP-1 β supported higher levels of mucosal S-IgA antibodies (Lillard *et al.*, 2003).

Saponin derivatives

QS-21 is a highly purified complex triterpene glycoside isolated from the bark of the *Quillaja saponaria* Molina tree (Kensil and Kramer, 1998). This molecule promotes both humoral and CMI responses when added to systemic

vaccine formulations and has now been tested in several parenteral vaccine formulations (Livingston *et al.*, 1997; Kensil and Kramer, 1998). QS-21 was reported to promote both systemic and mucosal immunity to a nasal DNA vaccine (Sasaki *et al.*, 1998). Others have shown that QS-21 also acts as adjuvant when given to mice by the oral route (Boyaka *et al.*, 2001). Interestingly, low oral QS-21 doses promoted mucosal S-IgA Ab responses, while no S-IgA antibodies were induced by high oral QS-21 (Boyaka *et al.*, 2001). On the other hand, stronger Th1-type responses were seen after immunization with high oral QS-21 doses (Boyaka *et al.*, 2001). It is possible that the mild detergent activity of QS-21 and related saponin derivatives such as QS-7 (Kensil and Kramer, 1998) may enhance immune responses by facilitating antigen uptake. This point and mechanisms by which saponin derivatives promote mucosal immunity and control CD4⁺ Th cell subset differentiation remain to be determined.

Mucosal vaccine delivery systems

Empirical experience with mucosal immunization has resulted in the commonly held view that repeated administration of high doses of antigen is required to achieve the levels of immune response induced by systemic immunization. This conclusion is physiologically justifiable since the primary function of the mucosal immune system is to prevent overstimulation of the entire immune system. It does so by eliminating mucosally applied antigens with denaturing acids, degradative enzymes, and other factors of innate immunity or through intestinal peristalsis and ciliary movement on epithelia in the respiratory tract. As a result of the concerted interactions of such factors, little antigen remains to be absorbed and thereby to stimulate inductive sites for mucosal and systemic immune responses. Mucosal vaccinologists have devised delivery systems which circumvent such obstacles. The comparative advantages and disadvantages of mucosal antigen delivery will be dealt with here, and the details are covered in ensuing chapters. Furthermore, it should be emphasized that most results obtained have been generalized in animal models rather than in humans, with all unavoidable limitations.

M cell targeting delivery systems

The ability of some viral outer capsid proteins to bind to M cells covering mucosal inductive sites has recently been exploited to devise a delivery systems protein, DNA, and possibly polysaccharide vaccines (see Chapter 7). In this regard, reoviruses are nonenveloped, double-stranded RNA icosahedral viruses composed of three serotypes (Schiff and Fields, 1990). The most widely studied serotypes, 1 and 3, enter the host via M cells in the FAE. Binding to M cells is mediated via the C-terminus of the minor outer capsid protein sigma one ($p\sigma 1$) (Mah *et al.*, 1990). The $p\sigma 1$ subunit is 45 kDa and polymerizes via its N-terminus to form a stable tetramer (Bassel-Duby *et al.*, 1987). Incorporation of $p\sigma 1$ into liposomes was shown to allow their binding to mouse L cells and rat Peyer's patches (Rubas *et al.*, 1990). Likewise, the $p\sigma 1$ can bind to NALT M cells (Wu *et al.*,

2000, 2001). Of major interest for mucosal vaccine development, immunization with DNA complexed to $p\sigma 1$ could overcome the low immunogenicity of naked DNA and promote elevated S-IgA and plasma IgG antibody responses (Wu *et al.*, 2001). Protein and polysaccharide vaccine antigens could be linked to $p\sigma 1$ for optimal targeting of M cells and mucosal inductive sites. However, extrapolation of this strategy for human vaccination will first require that we determine the serotype of reovirus with the best affinity for human M cells.

Inert vaccine delivery systems

Since Ags are more immunogenic in particulate form than in solutions and are vulnerable to antigen-degrading enzymes and acids, they have often been incorporated into vehicles that are by themselves nontoxic and nonimmunogenic but which protect vaccine material from degradation, enhance their uptake from mucosal surfaces, and may exhibit some adjuvant effect (see Chapter 55). A few examples are discussed next.

Gelatin capsules

Gelatin capsules coated with substances (*e.g.*, cellulose acetate phthalate) that dissolve at alkaline pH in the intestine but not at acid pH in the stomach have been used for oral delivery of bacterial and viral vaccines in several studies performed in humans. Although vaccines given in enteric-coated capsules have not been rigorously shown to be more effective than free antigens, influenza virus exposed to acid pH is lowly immunogenic, while it remains intact when the virus is placed in enteric-coated gelatin capsules, where the drop in pH is greatly diminished (Moldoveanu *et al.*, 1993).

Mucoadhesive polymers

Mucoadhesive polymers that adhere to mucosal surfaces extend the time of the exposure of vaccines and thus facilitate the induction of immune responses. Compounds such as highly viscous inert polysaccharide eldexomer and carboxymethyl cellulose were used for oral, nasal, or vaginal delivery of antigens such as influenza virus or CT-B and could promote both local mucosal and systemic immune responses. Other compounds that have been considered are carbapol, polycarboxophil, sodium alginate, and hydroxypropyl cellulose, which are used in medicine for delivery of drugs to combat diarrhea and constipation and to promote wound healing.

ISCOMs

Immunostimulating complexes (ISCOMs) are cage-like particles generated after addition of cholesterol to the Quil A from the bark of the *Quillaja saponaria* Molina tree. ISCOMs are effective oral delivery systems that promote mucosal and systemic immunity. More recently, ISCOMs containing a fusion protein comprising the OVA (323–339) peptide epitope linked to CTA1-DD were highly immunogenic when given in nanogram doses by the oral or nasal routes (Mowat *et al.*, 2001). Interestingly, ISCOMs

containing the enzymatically inactive CTA1-R7K-DD mutant protein were much less effective, suggesting that at least part of the activity of the combined vector requires the ADP-ribosylating property of CTA1 (Mowat *et al.*, 2001).

Biodegradable microspheres

Biodegradable microspheres composed of antigens incorporated into polymers of lactic and glycolic acid have been used far more extensively in mucosal vaccinology than any other mucosal delivery system (Mestecky *et al.*, 1997). Soluble proteins, viral and bacterial glycoproteins, and viruses (*e.g.*, influenza and SIV) have been given as microencapsulated vaccines orally or intratracheally to humans, monkeys, and rodents in many studies. Microspheres are to be preferred to most of the other delivery systems because of their stability, which allows them to protect incorporated antigens from acids and enzymes; the ease with which their size and rapidity of biodegradation can be modified during the fabrication process; their nontoxicity and resulting unparalleled record of safety; and their biodegradability.

Transgenic plants

Novel molecular methods have allowed the production of subunit vaccines in transgenic plants (see Chapters 58 and 59). Plants can be engineered to synthesize and assemble an adjuvant and one or more antigens which retain both T and B-cell epitopes (Haq *et al.*, 1995; Tacket *et al.*, 1998; Yu and Langridge, 2001). The feeding of transgenic potato tubers was shown to induce systemic and mucosal immune responses in mice (Haq *et al.*, 1995) and in humans (Tacket *et al.*, 1998). In order to circumvent potential denaturation of antigen during cooking, recombinant plants that do not require cooking such as bananas and tomatoes are being developed.

Live recombinant vaccine delivery systems

Many bacterial and viral vectors (*Salmonella*, *E. coli*, mycobacteria, lactobacilli, polio-, adeno-, rhino-mengo-, influenza, vaccinia, and canarypox viruses) have been used with variable success in animal models (see Chapters 56 and 57). A recombinant bacterial vector (*i.e.*, *Salmonella typhi* Ty21A) is licensed in the United States for use as an oral vaccine (Table 47.1). In June 2003, a cold-adapted live influenza vaccine (*i.e.*, FluMist) was approved as a nasal vaccine for humans (Table 47.1). Despite these significant advances, further studies will be required to determine optimal mucosal immunization protocols and to enhance the expression of future live recombinant vaccines while diminishing responses to the vector to prevent its elimination by immune mechanisms. In fact, vectored delivery has been limited to former pathogens that have been subjected to attenuating mutations or genomic deletions. Nonpathogenic strains of bacteria such as lactobacilli are being evaluated as vaccine delivery systems. In most instances, host immune responses develop both to recombinant vectors and to the transgenes. The use of different recombinant vectors for priming and boosting could help reduce potential immunity to the vectors.

IMMUNE RESPONSE VERSUS MUCOSAL TOLERANCE

Extensive experiments performed in animals clearly indicate that oral or nasal exposure to certain antigens (*e.g.*, myelin basic protein, ovalbumin, cartilage type II collagen) induces a state of unresponsiveness in the systemic compartment upon systemic immunization (see Chapter 27). Recently, the existence of this phenomenon was documented in humans immunized orally (Husby *et al.*, 1994; Kraus *et al.*, 2004) or nasally (Waldo *et al.*, 1994) with keyhole limpet hemocyanin (KLH). Subsequent systemic immunization with the same antigen clearly demonstrated boostable mucosal and systemic humoral immune responses but suppressed T-cell proliferation and delayed-type hypersensitivity (DTH) reactions (so called split tolerance). Consequently, it is unlikely that orally or nasally applied vaccines would induce the state of unresponsiveness in the humoral arm of the immune response. Considering the fact that all vaccines used so far in human medicine exhibit their protective effect through the induction of specific antibodies rather than CTLs, the possibility of inducing tolerance by mucosal exposure is minimal. Nevertheless, the suppression of Th1 and perhaps Th2 responses would be counterproductive in immunization strategies whose aim is to induce protective T cell-mediated responses (*e.g.*, to HIV). However, it should be emphasized that the suppression of T cell-mediated responses occurs only when the same antigen is administered by the mucosal route to the previously naïve animal. In sharp contrast, initial systemic priming and ensuing B- and T-cell responses cannot be suppressed by subsequent mucosal immunization. This fact of basic impact is unfortunately often disregarded. Therefore, it has been assumed that ongoing systemic immune responses, particularly of autoimmune character, can be suppressed by mucosal exposure to antigens that elicited preceding systemic responses. Contrary to this assumption, Chase (1946) dramatically demonstrated that the cell-mediated responses induced by systemic immunization of animals with a hapten-carrier cannot be suppressed by oral administration of the same antigen. In the same vein, DTH reactions to poison ivy or poison oak cannot be suppressed by oral administrations of dried leaves or their various extract in humans (for reviews see Stevens, 1945; Klingman, 1958). More recent studies have clearly demonstrated that ongoing vigorous responses induced by systemic immunization with autoantigens are refractory to suppression by nasal or oral administration of the same antigen (for review see Czerkinsky, 1999). These studies were recently extended to humans and indicated that ongoing humoral and cellular responses induced by systemic immunization with KLH cannot be suppressed by subsequent extended ingestion of large doses of KLH (Elson, C.O., University of Alabama at Birmingham, personal communication).

In summary, the sequence of systemic priming followed by mucosal boosting provides vigorous humoral and cellular immune responses in both mucosal and systemic compartments, with minimal danger of inducing tolerance in the cell-mediated arm of the immune response.

Mucosal therapeutic vaccines against autoimmune and inflammatory infectious diseases

It is now well established that adjuvants are required for effective induction of mucosal immunity. Less appreciated is the fact that delivery systems and/or adjuvants are also needed to enhance mucosal tolerance for effective immunotherapeutic applications. Thus far, the most promising vehicle for inducing mucosal tolerance is CT-B. Thus, mucosal delivery of CT-B chemically linked or genetically fused to various autoantigens is generally a more effective method for inducing tolerance than mucosal administration of autoantigen alone. For example, oral or nasal treatment with low-dose CT-B chemically conjugated or genetically linked (fusion proteins) to myelin basic protein (Sun *et al.*, 2000), insulin (Bergerot *et al.*, 1997), or collagen II (Tarkowski *et al.*, 1999) and/or selected peptides derived from these antigens could totally or markedly suppress the development of experimental autoimmune encephalitis (EAE), type I diabetes, or collagen-induced arthritis, respectively. In addition, prolonged oral treatment with low doses of allergen conjugated to CT-B markedly suppressed IgE antibody responses and allergic reactions in sensitized mice (Rask *et al.*, 2000). More recently, mucosally induced uveitis could be prevented in rat by oral administration of CT-B linked with the uveitogenic peptide (aa 336-351) derived from human heat-shock protein (HSP) 60 (Phipps *et al.*, 2003).

The mechanisms underlying the enhancement of oral tolerance by CT-B-Ag conjugates are probably complex and only partially understood. As for induction of mucosal immunity, DCs appear to also play a critical role in mucosal tolerance (Viney *et al.*, 1998). Thus, priming of DCs may constitute a major step in the induction of immune responses to mucosally delivered antigens. The development of active adaptive responses versus tolerance could then be dictated by additional stimuli including cytokines and costimulatory molecules.

For example, treatment with different CT-B-Ag conjugates or fusion proteins was reported to promote actively tolerogenic APCs and TGF- β -secreting suppressive-regulatory T cells in mucosal tissues and draining lymph nodes (Sun *et al.*, 2000). Oral feeding of CT-B was shown to both prevent and cure Th1-driven experimental colitis in mice, through reduction of IL-12 production within the large intestine (Boirivant *et al.*, 2001). Furthermore, tolerization was also shown to downregulate chemokines, including RANTES (Sun *et al.*, 2000), a chemokine that stimulates adaptive immunity (Lillard *et al.*, 1999).

SAFETY OF MUCOSAL VACCINES, ADJUVANTS, AND DELIVERY SYSTEMS

The watery diarrhea induced by native enterotoxin is the major safety concern identified thus far for oral vaccines. As seen above, nontoxic mutant has been developed to overcome this toxicity. Unfortunately, nontoxic enterotoxin mutants are generally poor adjuvants for oral vaccines,

although they often retain their adjuvanticity for nasally coadministered vaccine antigens. While reviewing the safety, immunogenicity, and efficacy of oral rhesus-human reassortant vaccine candidates, a Rotavirus Working Group of the Advisory Committee on Immunization Practices (ACIP) of the Centers for Diseases Control and Prevention (CDC) noted 5 cases of intussusception among 10,054 vaccinees, 3 of which occurred during the first week postvaccination, and 1 case among 4,633 placebo recipients (Margaret *et al.*, 2000). While none of these cases had been judged by the clinical study investigators to be directly attributable to the vaccine, the product was withdrawn from the market. Thus, better methods are needed to analyze and predict potential toxicity of oral vaccines.

The nasal cavity is anatomically close to the olfactory nerves/epithelium (ON/E) and olfactory bulbs (OBs). Recent studies examined whether, in addition to NALT, CT as adjuvant could target these tissues of the central nervous system. Both CT and its B subunit (CT-B) entered the ON/E and OB and persisted for 6 days, although neither molecule was present in NALT beyond 24 hours after nasal delivery (van Ginkel *et al.*, 1999). This uptake into olfactory regions was monosialoganglioside (GM1)-dependent. Nasal vaccination with ^{125}I -TT together with unlabeled CT as adjuvant resulted in uptake into the ON/E but not the OB, whereas ^{125}I -TT alone did not penetrate into the CNS. This study suggested that GM1-binding molecules like CT target the ON/E and are retrograde-transported into the OB and may promote uptake of vaccine proteins into olfactory neurons. This raises concerns about the role of GM1-binding molecules that target neuronal tissues in mucosal immunity (van Ginkel *et al.*, 1999). The targeting of CNS tissues by nasally administered bacterial enterotoxins is possibly related to a higher incidence of Bell's palsy (facial paresis) among volunteers of a nasal vaccination trial with native LT as mucosal adjuvant. Thus, of the serious adverse events reported for approximately 3,600 subjects participating in a clinical (safety) trial for a nonliving nasal flu vaccine (Nasalflu) in 2000, there were 9 cases of Bell's palsy and 1 of trigeminal neuralgia that developed into facial paresis. Furthermore, five cases of Bell's palsy were spontaneously reported from among approximately 90,000 recipients of Nasalflu, and the vaccine was withdrawn from the market (www.niaid.nih.gov/dmid/enteric/intranasal.htm; Table 47.1). The recent approval of the trivalent FluMist cold-adapted live influenza vaccine demonstrates that the nasal route remains a viable choice for delivery of carefully devised mucosal vaccines.

SUMMARY AND PERSPECTIVES

Like HIV and AIDS some 20 years ago, the recent worldwide outbreak of severe acute respiratory syndrome (SARS) dramatically reminded us that infectious diseases remain a major global threat that is no longer restricted to developing countries or selected populations. The newly discovered coronavirus responsible for SARS (Kuiken *et al.*, 2003)

infects the host via mucosal tissues of the respiratory tract, where both mucosal antibodies and CMI responses could provide the host with lifesaving protection. The cases of inhalational anthrax related to a bioterrorism attack in the United States during October and November 2001 (Jernigan *et al.*, 2001; Guarner *et al.*, 2003) further demonstrated the importance of mucosal tissues as portals for entry of pathogenic agents used as bioterrorism agents. In this regard, transmission of all the Category A bioterrorist agents/diseases listed by the CDC occurs by either inhalation or ingestion. Our understanding of mechanisms by which adjuvants control mucosal antibody and CMI responses has greatly improved over the past decade. In fact, we now know that pattern recognitions, together with innate factors such as cytokines and chemokines and the APC function of DCs, are crucial for initiation of mucosal immunity. New and improved delivery systems will be critical for use with new mucosal vaccines such as the nasal FluMist vaccine. The ADP ribosyltransferase activity of bacterial enterotoxins has been the only potential toxicity investigated in mucosal vaccines. The recent knowledge that nasal vaccines can potentially bind to neurons has fostered research on the safety of mucosal vaccines and should help in the design of safer vaccines to be administered by the nasal or other mucosal routes. The recently approved FluMist vaccine could then be the first of a long list of new-generation mucosal vaccines.

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