A revisit of mucosal IgA immunity and oral tolerance

Kohtaro Fujihashi, Hirotomo Kato, Frederik W. van Ginkel, Toshiya Koga, Prosper N. Boyaka, Raymond J. Jackson, Rie Kato, Yukari Hagiwara, Yuri Etani, Iwao Goma, Keiko Fujihashi, Hiroshi Kiyono and Jerry R. McGhee

The Departments of Oral Biology and Microbiology, The Immunobiology Vaccine Center, The University of Alabama at Birmingham, Birmingham, Alabama, USA

Fujihashi K, Kato H, van Ginkel FW, Koga T, Boyaka PN, Jackson RJ, Kato R, Hagiwara Y, Etani Y, Goma I, Fujihashi K, Kiyono H, McGhee JR. A revisit of IgA immunity and oral tolerance. Acta Odontol Scand 2001;59:301–308. Oslo. ISSN 0001-6357.

Induction of mucosal immunity by oral immunization with protein antigen alone is difficult: potent mucosal adjuvants, vectors, or other special delivery systems are required. Cholera toxin (CT) has been shown to be an effective adjuvant for the development of mucosal vaccines and, when given with vaccine, induces both mucosal and systemic immune responses via a Th2 cell-dependent pathway. However, and in addition to potential type-I hypersensitivity, a major concern for use of mucosal adjuvants such as CT is that this molecule is not suitable for use in humans because of its inherent toxicity. When we examined the potential toxicity of CT for the central nervous system, both CT and CT-B accumulated in the olfactory nerves/epithelium and olfactory bulbs of mice when given by the nasal route. The development of effective mucosal vaccines for the elderly is also an important issue; however, only limited information is available. When mucosal adjuvanticity of CT was evaluated in aged mice, an early immune dysregulation was evident in the mucosal immune system. The present review discusses these potential problems for effective mucosal vaccine development. Tolerance represents the most common and important response of the host to environmental antigens, including food and commensal bacterial components, for the maintenance of an appropriate immunological homeostasis. We have examined whether Peyer patches could play a more important role for the maintenance of oral tolerance. Using Peyer patch-null mice, we found that mice lacking this gut-associated lymphoid tissue retained their capability to produce secretory IgA antibodies but did not develop normal oral tolerance to protein antigens. □ *Aging; mucosa; tolerance; toxicity; vaccines*

Kohtaro Fujihashi, Department of Oral Biology, Bevill Biomedical Research Building, Room 761, School of Dentistry, Immunobiology Vaccine Center, The University of Alabama at Birmingham, 845 19th St. South, Birmingham, AL 35294-2170. Tel: +1 205 934-1951, fax: +1 205 975-4431, e-mail: Kohtaro_Fujihashi@micro.microbio.uab.edu

The mucosal immune system is anatomically and functionally divided into sites. Foreign antigens are encountered and taken up for initiation of immune responses at the inductive sites. The more diffuse collections of B and T lymphocytes, differentiated plasma cells, macrophages (Mφ) and other antigen-presenting cells (APCs), eosinophils, basophils, and mast cells in the lamina propria/exocrine gland interstitium constitute the effector sites for mucosal immunity. This network is highly integrated and finely regulated, and the outcome of mucosal tissue encounters with foreign antigens and pathogens can range from mucosal and serum antibody (Ab) responses and T-cell-mediated immunity, on the one hand, to systemic anergy and mucosally encountered antigen, a response commonly termed mucosally induced tolerance, on the other (1, 2).

Mucosal inductive sites include the Peyer patches as gut-associated lymphoid tissues (GALT) and the Waldeyer ring of tonsils and adenoids as nasopharyngeal-associated lymphoid tissue (NALT), which collectively comprise a mucosa-associated or MALT network for continuous supply of memory B and T cells to mucosal effector sites (1, 2). The homing of lymphocytes from inductive to effector mucosal tissues is the cellular basis for the common mucosal immune system (CMIS), in which either nasal or oral vaccination induces mucosal immunity in distant multiple effector sites (1, 2) (Fig. 1). The MALT consists of both T- and B-cell-enriched areas (the latter of which contains a high percentage of surface IgA-positive ($sIgA^+$) B cells) and APCs necessary for the induction of specific immune responses. Covering the MALT is a specialized epithelium (FAE) that contains a subset of differentiated epithelial cells termed microfold or M cells, in addition to columnar epithelial cells and lymphoid cells (3). The FAE M cell plays an important role in the initial phase of induction of mucosal immune responses by sampling antigens from the lumen of the gut or nasal passages and transporting the antigen intact to the underlying APCs for initiation of the immune response. Antigen-activated and memory B- and T-cell populations then emigrate from the inductive environment via lymphatic drainage, circulate through the bloodstream, and home to mucosal effector sites (Fig. 1). These effector sites include more diffuse tissues where antigen-specific T and B lymphocytes ultimately reside and perform their respective functions (that is, T-cell help for Ab responses and cell-mediated immunity (CMI) for cytotoxic T lymphocyte (CTL) induction and regulatory functions or Ab synthesis, respectively) to protect mucosal surfaces.

Oral tolerance is a unique immune reaction characterized by the fact that experimental animals fed large quantities of protein antigen (Ag) become refractory or

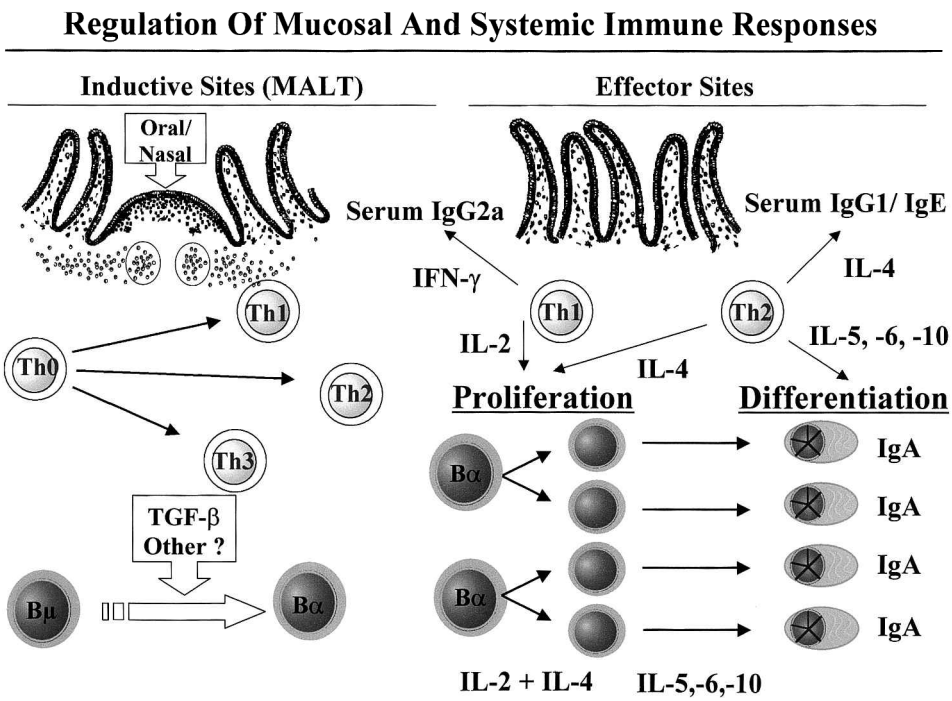

Fig. 1. The role of inductive sites for mucosal IgA responses. Antigen uptake by M cells occurs in mucosa-associated lymphoreticular tissue (MALT) (gut-associated lymphoreticular tissue, bronchus (B)-associated lymphoreticular tissue, and nasopharyngeal-associated lymphoreticular tissue) and results in the initial induction of the immune response. Antigen-sensitized, precursor $sIgA^+$ B cells, $CD4^+$ Th cells, and $CD8^+$ cytotoxic T lymphocytes (CTLs) in GALT leave via draining lymphatics and migrate to mesenteric lymph nodes and then into the thoracic duct to reach the bloodstream. These migrating cells enter the IgA effector sites, where terminal differentiation, IgA synthesis, and transport of S-IgA occur. This induction in MALT and exodus of cells to effector sites is termed the common mucosal immune system. IFN = interferon; IL = interleukin; TGF = transforming growth factor.

have a diminished capacity to develop an immune response when re-exposed to that same Ag introduced by the systemic route (for example, by injection) (4–8). This unique response is an important natural physiological mechanism whereby the host avoids development of cell-mediated immunity (CMI) or delayed-type hypersensitivity (DTH) and formation of potentially harmful Abs—that is, IgE to many ingested food proteins and similar antigens (5).

Although several possible mechanisms (B-cell tolerance, anti-idiotypic Ab, intestinal antigen-processing events resulting in a tolerogen, and conventional APCs) have been shown to be involved in the induction of oral tolerance (8), most studies now suggest that T cells are the major cell type involved in the induction of oral tolerance (6, 8–15). In earlier work, it was shown that systemic unresponsiveness was induced by adoptive transfer of T cells from rats orally fed bovine serum albumin (16). Later, a large number of studies showed that oral immunization with protein antigen induced $CD4^+$ Th cells in mucosa-associated tissues that supported IgA responses, whereas suppressor T cells were induced in systemic compartments such as spleen that downregulated antigen-specific IgM, IgG, and IgE Ab responses (17–23). Furthermore, the helper T cells for IgA responses tended to remain in Peyer patches, whereas the suppressor T cells were found to migrate into the systemic compartment (for example, the spleen). These observations were considered to be logical explanations for cellular mechanisms of oral tolerance in which Peyer patch-derived $CD4^+$ Th cells supported IgA responses, whereas splenic T suppressor cells induced systemic unresponsiveness. It is now generally agreed that a functional suppressor mechanism exists for the down-regulation of systemic immune responses; however, the nature and properties of these suppressor T cells are disputed.

Adjuvants are required for mucosal immunization

The major enterotoxins produced by *Vibrio cholerae* and *Escherichia coli* are cholera toxin (CT) and heat-labile toxin I (LT), respectively. Mucosal exposure of native (n) CT and nLT, which are both immunogenic, results in secretory

IgA (S-IgA) and serum Ab, which are almost entirely restricted to CT-B or LT-B (24). Both nCT and nLT are potent mucosal adjuvants for co-administered unrelated proteins when given by oral, nasal, or even parenteral routes (24–26). Recent studies have shown that induction of maximal mucosal S-IgA and serum IgG Ab responses correlates directly with the presence of antigen-specific $CD4^+$ Th cells secreting interleukin (IL)-4 and IL-5 in mice orally immunized with protein antigen and nCT as adjuvant (25). Further analysis showed that nCT elicits adjuvant responses by inducing antigen-specific $CD4^+$ Th2-type cells, which in turn produce high levels of IL-4, IL-5, IL-6, and IL-10. These Th2 cells support subsequent development of systemic IgG1 and IgG2b subclasses, IgE, and mucosal S-IgA Ab responses (27). Oral immunization with nLT results in serum IgM, IgG1 = IgG2a, less IgG2b, and mucosal S-IgA Ab responses, which are associated with $CD4^+$ Th1- and IL-4-independent Th2-type responses with interferon (IFN)-γ, IL-5, IL-6, and IL-10 production (28).

It is now well known that both nCT and nLT cause severe diarrhea in humans and thus are not suitable for use as mucosal adjuvants. Earlier studies have attempted to dissociate the diarrheagenicity and adjuvanticity of these two molecules. It was first shown that a mutant of LT, termed E112K (29), which contains a single amino acid substitution in the adenosine diphosphate (ADP)-ribosyltransferase-active center (30), was nontoxic but also lacked adjuvanticity (31). This initial study evaluated LT E112K as an oral adjuvant, whereas more recent studies now suggest that this mutant is an effective adjuvant when given by the nasal route (32). In addition, it was reported that single amino acid substitution mutants of LT, R7K (33), and R192G (34) are nontoxic but retain adjuvant properties when co-administered with protein by the nasal or oral routes, respectively (33, 34). Another LT mutant, S61K, was shown to be without toxicity and a poor mucosal adjuvant (35, 36). With regard to CT, two mutant CT (mCT), designated S61F and E112K, which harbor single amino acid substitutions in the ADP-ribosyltransferase-active center complex, lack ADP-ribosyltransferase activity and diarrheagenicity (37). Both mCTs are effective adjuvants and are comparable to nCT when given parenterally (37) or nasally (38).

Potential toxicity associated with nasal delivery of cholera toxin as mucosal adjuvant

Currently, mucosal immunization consists of either oral (GI tract) or nasal (upper respiratory tract) delivery. In fact, many new vaccines are being tested by both routes to determine which induces immune responses via the CMIS most effectively. In many cases, nasal immunization is more effective and in general requires smaller vaccine doses with less adjuvant. Most current protocols instil vaccine into each nostril (usually 5–10 μl/nostril), and normal inhalation results in effective delivery of vaccine, presumably into NALT. However, one must consider the potential for nasal vaccines to enter the central nervous system (CNS) because of the anatomical proximity of the olfactory nerves/epithelium (ON/E) and olfactory bulbs (OB) to the brain.

This potential for neurotoxicity has major implications for the use of these mucosal adjuvants in humans. However, no major side effects were reported after nasal application of CT-B to 12 IgA nephropathy patients (39). It is not yet clear whether CT-B alone would show potential CNS-related problems or, alternatively, whether the A subunit would also be required. An abstract at the Cold Spring Harbor Vaccine meeting in December 1999 (Molecular Approaches to Vaccine Design) showed that LT and detoxified LT mutants also targeted the CNS of mice after intranasal application (40). This confirms that both nCT and nLT derivatives display similar characteristics following nasal application and their targeting to the CNS raises serious concerns.

To further explore the potential toxicity of nCT for the CNS, we have recently examined trafficking of nasally administered CT in the mouse model. In the initial study, ^{125}I-labeled CT-B was given nasally, and neuronal tissues (ON/E, OB, and brain) were analyzed for the presence of ^{125}I-CT-B. The radioactivity in the ON/E peaked at 6 h, reached a plateau, and remained there for 6 days (41), whereas OB peaked at 15 min and remained relatively constant over time. Furthermore, the ON/E and OBs were the only tissues that showed this profile of ^{125}I-CT-B accumulation. The distribution of ^{125}I-labeled CT after nasal application followed similar kinetics in NALT, ON/E, and OB when compared with ^{125}I-CT-B. These results indicate that neuronal binding is a characteristic of nCT.

To determine whether nCT, when used as mucosal adjuvant, redirects protein vaccines into neuronal tissues, ^{125}I-tetanus toxoid (TT) was co-administered with or without nonlabeled nCT nasally. The ^{125}I-TT distribution in various tissues was compared with Ag given with or without nCT. A delay in clearance of ^{125}I-TT was observed in both lymphoid and CNS tissues of mice given ^{125}I-TT with nCT. Co-administration of CT showed increased levels of ^{125}I-TT at 24 and 48 h, and this progressively decreased over the course of 6 days (41). The experiments show that adjuvant co-administration may modify protein vaccine uptake.

Impaired mucosal immunity in aged mice

The precise nature of mucosal immune responses that occur in the elderly remain poorly defined. To gain more information on this important issue, we have recently compared the mucosal immune responses of aged and young adult mice immunized weekly with three oral doses of 1 mg of ovalbumin (OVA) and 10 μg of nCT as mucosal adjuvant. Our previous studies have shown that mucosal immunization with protein Ags co-administered with nCT

Table 1. Comparison of antigen-specific antibody (Ab) responses in aged and young adult mice

Route of immunization	Samples	OVA-specific Ab responses* (IgG for serum, IgA for fecal extracts)		CT-B-specific Ab responses* (IgG for serum, IgA for fecal extracts)	
		6–8 weeks	12–14 months	6–8 weeks	12–14 months
Oral	Serum	18	12.5	19	12.5
	Fecal extracts	5.5	<3	7	<3
Subcutaneous	Serum	15.5	10.5	19	18

OVA = oval bumin; CT = cholera toxin.
* End-point titers were expressed as the last dilution yielding an optical density at 414 nm (optical density, 414) of >0.1 units above negative control values after a 15-min incubation.

as mucosal adjuvant elicited Th2-type cytokines, especially IL-4, and mediated mucosal and systemic immune responses to protein Ag and to nCT itself (25, 27). This mucosal immunization regimen is thus ideal to address the effects of aging on the mucosal immune system of mice. Our study has shown that dysregulation occurs in the mucosal immune system as early as 12–14 months of age, whereas systemic immunity remains essentially normal (42). Thus, mice more than 1 or 2 years of age showed reduced levels of Ag-specific mucosal or systemic immune responses on day 21 (42) (Table 1). An Ag-specific B-cell ELISPOT assay confirmed these results at the cellular level. When the Ag-induced cytokine responses were examined at both protein and mRNA levels, $CD4^+$ T cells from spleen and Peyer patches of young adult mice showed increased levels of IL-4 production; however, these cytokine responses were significantly diminished in aged mice (42). In contrast to mucosal immunization, subcutaneous immunization of mice with OVA plus CT resulted in impaired OVA-specific but intact CT-B-specific immune responses in 12- to 14-month old mice (Table 1), although the responses to both Ags were depressed in 2-year-old mice.

Another novel finding of our study was that parenteral immune responses in 1-year-old mice immunized subcutaneously with OVA and CT showed a less marked immune deficiency than did those of mice more than 2 years old (42). In this regard, serum IgG anti-CT-B Ab responses in 1-year-old mice were comparable to those of young adult mice, even though anti-OVA IgG Ab responses were essentially absent. Further, intact OVA-specific $CD4^+$ T-cell proliferative responses were seen in 3 of 16 1-year-old mice (42). In contrast, mucosal immune responses including both OVA- and CT-B-specific Ab and cytokine responses induced by oral OVA and CT in 1-year-old mice were markedly reduced and were comparable to those seen in 2-year-old mice. In addition, systemic immune responses were also diminished in 1-year-old mice given oral OVA and CT. When one considers the common mucosal immune system, mucosal inductive sites such as the Peyer patches must play a central role in the induction of antigen-specific immune responses in both mucosal and systemic tissues. Thus, the decreased IgG Ab responses in 1-year-old mice immunized with oral OVA plus CT may simply be due to the impaired mucosal immune system in these mice. To support this, systemic immunization resulted in intact OVA-specific T-cell responses and CT-B-specific Ab responses in 1-year-old mice. These results indicate that age-associated alterations arise in the mucosal immune system earlier than in the parenteral immune compartment.

The central importance of the mucosal immune system as a first line of defense against numerous pathogens has been well established. For example, pathogens that are encountered after ingestion or inhalation may subsequently colonize the GI or upper respiratory tracts (1). Both oral and nasal immunizations have been shown to effectively induce mucosal immune responses at the mucosal barrier itself (1). We feel that our findings presented here may be of importance in efforts to develop effective mucosal vaccines for the elderly. For example, vaccines to prevent influenza and *Streptococcus pneumoniae* pneumonia are less effective in the elderly. Thus, one may postulate that induction of Ag-specific mucosal immunity in both middle-aged and aged individuals would be difficult to achieve and may explain vaccine failures. In addition, innate immunity also plays important roles in host defense and in maintaining immune homeostasis.

A revisit of mucosal immune responses in oral tolerance

On the basis of the initial finding that oral administration of a streptococcal Ag or OVA to mice simultaneously resulted in suppression of Ag-specific systemic immune responses in the presence of salivary S-IgA Ab responses (7), this dual state of systemic unresponsiveness and mucosal S-IgA immunity was dubbed oral tolerance (1, 7). Most studies in this area have tended to focus on systemic unresponsiveness after oral delivery and assume that mucosal S-IgA Abs are concurrently induced. Oral tolerance experiments have essentially been performed by initially feeding a protein Ag followed by systemic immunization with the same Ag given in complete Freund adjuvant (CFA). In these experiments a decrease in serum IgG and IgE Ab responses together with diminished T-cell

Table 2. Ovalbumin (OVA)-specific mucosal and systemic Ab responses in mice fed OVA or phosphate-buffered saline (PBS) before oral OVA plus cholera toxin (CT) immunization

Orally administered with	OVA-specific Ab Responses*		OVA-specific AFC/10^6 cells	
	IgA (fecal extracts)	IgG (serum)	IgA (lamina propria)	IgG (spleen)
PBS	7.5	15	~3500	~65
OVA	<3	<8	~500	<10

AFC = antibody-forming cells.
* End-point titers were expressed as the last dilution yielding an optical density at 414 nm (optical density, 414) of >0.1 units above negative control values after a 15-min incubation.

responses are considered to be the hallmarks of oral tolerance (8, 43). Systemic immunization with Ag with the potent adjuvant CFA did not enable us to determine whether tolerance extended to the mucosal compartment as well. It is now well established that parenteral immunization elicits systemic immunity in the absence of mucosal immune responses, and thus one cannot evaluate tolerance at the level of the GI tract mucosa with this type of regimen (1). When oral proteins are given with CT as a mucosal adjuvant, it has been shown that oral tolerance is not established, and Ag-specific mucosal IgA Ab responses are normally induced (24, 44). On the basis of these studies, mucosal immunization strategies using adjuvants such as CT and the related *Escherichia coli* LT have been developed (45). One of the advantages of mucosal immunization is that this mode can elicit both systemic and mucosal immune responses (1). In addition, this strategy also provides a unique way to address the mechanisms of induction and regulation of mucosal immune responses as manifested by S-IgA Ab production.

Oral tolerance has continued to be defined as systemic unresponsiveness with the maintenance of mucosal IgA Ab responses, and this goes back to studies performed more than 20 years ago (7). This finding preceded our knowledge that mucosal adjuvants such as CT can avert oral tolerance induction and induce potent mucosal IgA and systemic immune responses. Therefore, it was important to revisit this notion and to determine whether oral tolerance also influences mucosal immune responses, since oral tolerance has been mainly assessed by means of parenteral boosting with Ag in CFA. Further, studies should address whether potent mucosal adjuvants such as CT can reverse existing oral tolerance and induce both systemic and mucosal Ab responses.

To address these two important issues, mice were given OVA by gastric administration followed by an oral immunization protocol with OVA and CT as mucosal adjuvant. We have shown that a single high oral dose of OVA downregulates both systemic and mucosal immune responses, as assessed with a novel oral immunization strategy using OVA and CT as mucosal adjuvant (45). OVA-specific serum IgG, especially IgG1 subclass and IgA Ab responses, were diminished in OVA-fed mice when compared with mice given oral phosphate-buffered saline (PBS). Further, the numbers of OVA-specific IgG and IgA Ab-forming cells (AFC) in spleen were also dramatically reduced by OVA feeding before oral immunization. Of equal importance, mucosal IgA anti-OVA Abs were also reduced in OVA-fed mice subsequently challenged with OVA plus CT as mucosal adjuvant (Table 2) (45). The most direct test of the hypothesis that oral OVA would diminish mucosal IgA immunity came from studies assessing IgA AFCs in the GI tract. Dramatically reduced OVA but normal CT B-specific AFCs were seen in the intestinal lamina propria of mice fed OVA when compared with PBS-fed mice (Table 2). Significant reductions in $CD4^+$ T-cell proliferative responses and Th2-type cytokine production were also observed in mice fed OVA before oral immunization with OVA and CT as mucosal adjuvant. Furthermore, and perhaps more importantly, $CD4^+$ T cells from Peyer patches of OVA-fed mice subsequently orally immunized with OVA plus CT showed unresponsiveness to OVA, whereas Peyer patch $CD4^+$ T cell from PBS-fed mice showed significant OVA-specific proliferative and Th2-type cytokine responses. These results indicate that oral administration of a soluble protein Ag blocks the induction of a CMIS-dependent mucosal immune response, including $CD4^+$ T-cell responses and IgA Ab production in the GI tract. Further, a single high-dose feeding of protein Ag induces a state of Ag-specific immune unresponsiveness in mucosal compartments, indicating that CT does not break already established tolerance either in mucosal or systemic immune compartments.

Are Peyer patches required for oral tolerance?

Although the Peyer patches have been mainly viewed as major mucosal inductive sites for the generation of antigen-specific IgA responses, these tissues may also be important sites for dispatching immune suppressive signals to continuously ingested antigens. Tolerance (as defined by systemic unresponsiveness) represents the most common and important response of the host to environmental antigens, including food and commensal bacterial components, for the maintenance of appropriate immunological homeostasis. It is also important to consider that induction of mucosal and systemic immunity by oral immunization with protein antigen alone is rather difficult and requires

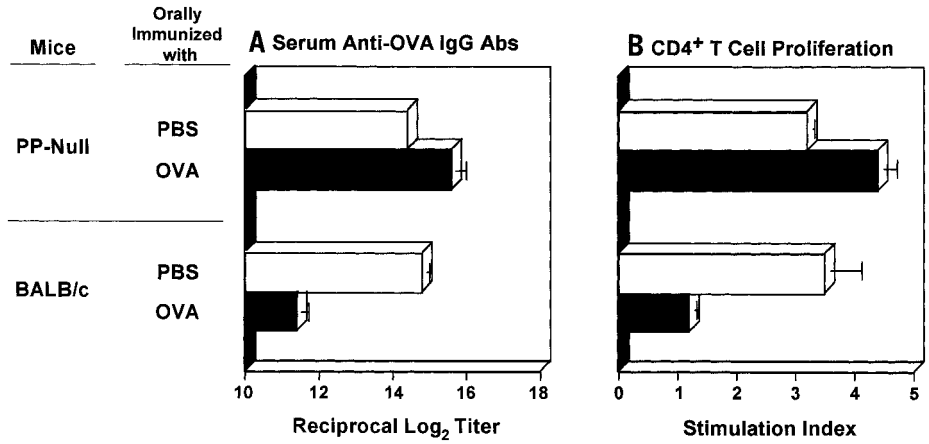

Fig. 2. (A) Ovalbumin (OVA)-specific IgG Ab responses in serum and (B) $CD4^+$ T-cell proliferative responses in mice from dams treated with lymphotoxin-β receptor (LTβR)-Ig fusion protein (PP-Null) or untreated BALB/c mice were then orally immunized with 25 mg of OVA or phosphate-buffered saline (PBS). Both groups of mice were challenged subcutaneously with 100 μg OVA in 100 μl complete Freund adjuvant 7 days after feeding. Fourteen days after subcutaneous OVA/CFA challenge, serum was assessed for OVA-specific IgG Ab responses. Splenic $CD4^+$ T cells were cultured with or without OVA for 5 days. The stimulation index was determined as cpm of wells with Ag/cpm of wells without Ag (controls).

use of potent mucosal adjuvants, vectors, or other special delivery systems. We have examined whether Peyer patches would play a more important role in the maintenance of oral tolerance.

Lymphotoxin signaling pathways are essential for lymphoid tissue development. Lymphotoxin-α knockout mice do not possess Peyer patches or associated lymph nodes (46, 47), and mice without lymphotoxin-β gene expression lack Peyer patches and peripheral lymph nodes but have mesenteric, sacral, and cervical lymph nodes (48–50). Aberrant development of peripheral lymphoid organs can be brought about by use of lymphotoxin-β receptor (LTβR) gene knockout mice (51) or administration of soluble LTβR-Ig fusion protein (52) or agonist antibody to the LTβR (53). Administration of LTβR-Ig during gestation also disrupts the development of peripheral lymph nodes and Peyer patches, whereas mesenteric, sacral, and cervical lymph nodes remain intact (52, 54). The latter system provides a unique and useful model to elucidate the role of Peyer patches in the induction of oral tolerance.

Thus, the immune responses in offspring of LTβR-Ig fusion protein-treated (Peyer patch-null) and control mice, given high oral doses of OVA before systemic challenge, were examined. In marked contrast to low OVA-specific IgG antibody (Ab) responses in serum and spleen of normal mice fed OVA, significant responses were seen in Peyer patch-null mice given oral OVA (Fig. 2) (55). Further, high T-cell proliferative and DTH responses were seen in Peyer patch-null mice given oral OVA before systemic challenge (Fig. 2) (55). Higher levels of $CD4^+$ T cell-derived IFN-γ, IL-4, IL-5, and IL-10 synthesis were noted in Peyer patch-null mice fed OVA, whereas OVA-fed normal mice had suppressed cytokine levels (55). These findings show that organized Peyer patches are required for oral tolerance to proteins. In support of this view, germ-free mice display a profound hypotropy of Peyer patches (56) and do not elicit systemic unresponsiveness when fed sheep erythrocytes for prolonged periods (57). Furthermore, absence of IgA Abs in mother's milk accelerates the development of Peyer patches in the neonate (58), perhaps due to the induction of tolerance to environmental antigens, which can easily invade the intestinal epithelium, in addition to a lack of passive immunity for protection. Nevertheless, it seems that Peyer patches have important dual functions for the induction of IgA responses and tolerance. Indeed, transforming growth factor (TGF)-β in Peyer patches has been shown to play a central role in the isotype switching of surface IgM to IgA B cells (59, 60), whereas this cytokine also has been detected in Peyer patches as a key factor in the induction of oral tolerance (61, 62). Although Peyer patches have dual functions in the induction of mucosal immunity and tolerance, it seems that establishment of oral tolerance by protein antigens is more dependent on the presence of organized GALT. Thus, it has been shown that intact mucosal IgA responses can be induced in Peyer patch-null mice orally immunized with OVA and CT as mucosal adjuvant (63). Further support for a requirement of GALT in oral tolerance induction was provided by in vivo treatment with *flt3* ligand. This treatment resulted in an expansion of dendritic cells in Peyer patches with enhanced oral tolerance induction (64). Taken together, these findings indicate that GALT provides downregulatory signals necessary to induce systemic unresponsiveness.

Conclusions

In the past 2 decades of the 20th century, studies in mucosal immunology have increased in a remarkable fashion. These studies have contributed to a better understanding of the cellular and molecular mechanisms of host defenses against infectious diseases. However, we still do not know the precise pathways in the mucosal immune system which comprise an important facet of host immunity. Thus, we should continue to seek safe mucosal adjuvant candidates based on the studies with CT adjuvanticity and toxicity. Further, it is important to revisit the oral tolerance concept in terms of its immunobiological mechanisms and involvement of immune cells and tissues. These studies will be of general interest to host immunity in the future.

Acknowledgements.—The authors thank Ms Sheila Turner and Ms Pamela Thomas for final preparation of this manuscript. This work is supported by U.S. Public Health Service grants AI 35932, DE 09837, AI 18958, DK 44240, AI 43197, DE 12242, and P30 DK 54781 and contract AI 65299.

References

1. McGhee JR, Kiyono H. Mucosal immunology. In: Paul WE, editor. Fundamental immunology. 4th ed. San Diego (CA): Academic Press; 1999. p. 909–45.
2. McGhee JR, Lamm ME, Strober W. Mucosal immune responses: an overview. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, editors. Mucosal immunology. 2nd ed. San Diego (CA): Academic Press; 1999. p. 485–506.
3. Kato T, Owen RL. Structure and function of intestinal mucosal epithelium. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, editors. Mucosal immunology. 2nd ed. San Diego (CA): Academic Press; 1999. p. 115–32.
4. Tomasi TB. Oral tolerance. Transplantation 1980;29:353–6.
5. Mowat AM. The regulation of immune responses to dietary protein antigens. Immunol Today 1987; 8:93–8.
6. Weiner HL, Friedman A, Miller A, Khoury SJ, Al-Sabbagh A, Santos L, et al. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu Rev Immunol 1994;12:809–37.
7. Challacombe SJ, Tomasi TB. Systemic tolerance and secretory immunity after oral immunization. J Exp Med 1980;152:1459–72.
8. Mowat AM, Weiner HL. Oral tolerance: physiological basis and clinical applications. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, editors. Mucosal immunology. 2nd ed. San Diego (CA): Academic Press; 1998. p. 587–618.
9. Mayer L. Oral tolerance: new approaches, new problems. Clin Immunol 2000;94:1–8.
10. Wardrop RM, Whitacre CC. Oral tolerance in the treatment of inflammatory autoimmune diseases. Inflamm Res 1999;48:106–19.
11. MacDonald, TT. T cell immunity to oral allergens. Curr Opin Immunol 1998;10:620–7.
12. Holt PG. Mucosal immunity in relation to the development of oral tolerance/sensitization. Allergy 1998;53:16–9.
13. Strobel S, Mowat AM. Immune responses to dietary antigens: oral tolerance. Immunol Today 1998;19:173–81.
14. Strober W, Kelsall B, Marth T. Oral tolerance. J Clin Immunol 1998;18:1–30.
15. Fujihashi, K, McGhee JR, Yamamoto M, Hiroi T, Kiyono H. Role of $\gamma\Delta$ T cells in the regulation of mucosal IgA response and oral tolerance. Ann NY Acad Sci 1996;778:55–63.
16. Thomas HC, Parrott MV. The induction of tolerance to a soluble protein antigen by oral administration. Immunology 1974;27: 631–9.
17. Kagnoff MF. Effects of antigen-feeding on intestinal and systemic immune responses. IV. Similarity between the suppressor factor in mice after erythrocyte-lysate injection and erythrocyte feeding. Gastroenterology 1980;79:4–61.
18. Kiyono H, Babb JL, Michalek SM, McGhee JR. Cellular basis for elevated IgA responses in C3H/HeJ mice. J Immunol 1980; 125:732–7.
19. Kiyono H, McGhee JR, Wannemuehler MJ, Michalek SM. Lack of oral tolerance in C3H/HeJ mice. J Exp Med 1982;155:605–10.
20. Mattingly JA, Waksman BH. Immunologic suppression after oral administration of antigen. I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. J Immunol 1978;121:1878–83.
21. Mowat AM, Lamont AG, Parrott DM. Suppressor T cells, antigen-presenting cells and the role of I-J restriction in oral tolerance to ovalbumin. Immunology 1988;64:141–5.
22. Richman LK, Graeff AS, Yarchoan R, Strober W. Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyer's patch after protein feeding. J Immunol 1981;126:2079–83.
23. Ngan J, Kind LS. Suppressor T cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. J Immunol 1978;120:861–5.
24. Elson CO, Ealding W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J Immunol 1984;132:2736–41.
25. Xu-Amano J, Kiyono H, Jackson RJ, Staats HF, Fujihashi K, Burrows PD, et al. Helper T cell subsets for immunoglobulin A responses: Oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. J Exp Med 1993;178:1309–20.
26. Clements JD, Hartzog NM, Lyon FL. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine 1988;6:269–77.
27. Marinaro M, Staats HF, Hiroi T, Jackson RJ, Coste M, Boyaka PN, et al. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J Immunol 1995;155:4621–9.
28. Takahashi I, Kiyono H, Marinaro M, Jackson RJ, Nakagawa I, Fujihashi K, et al. Mechanisms for mucosal immunogenicity and adjuvanticity of *Escherichia coli* labile enterotoxin. J Infect Dis 1996;173:627–35.
29. Tsuji T, Inoue T, Miyama A, Okamoto K, Honda T, Miwatani T. A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in a loss of its toxic activity. J Biol Chem 1990;265:22520–5.
30. Sixma TK, Pronk SE, Kalk KH, van Zanten BA, Berghuis AM, Hol WG. Lactose binding to heat labile enterotoxin revealed by X-ray crystallography. Nature 1992;355:561–4.
31. Lycke N, Tsuji T, Holmgren J. The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur J Immunol 1992;22: 2277–81.
32. Komase K, Tamura S-I, Matsuo K, Watanabe K, Hattori N, Odaka A, et al. Mutants of *Escherichia coli* heat-labile enterotoxin as an adjuvant for nasal influenza vaccine. Vaccine 1998;16:248–54.
33. Douce G, Turcotte C, Cropley I, Roberts M, Pizza M, Domenghini M, et al. Mutants of *Escherichia coli* heat-labile

toxin lacking ADP-ribosyltransferase activity act as nontoxic, mucosal adjuvants. Proc Natl Acad Sci USA 1995;92:1644–8.
34. Dickinson BL, Clements JD. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. Infect Immun 1995;63:1617–23.
35. Pizza M, Domenighini M, Hol W, Giannelli V, Fontana MR, Giuliani MM, et al. Probing the structure-activity relationship of *Escherichia coli* LT-A by site-directed mutagenesis. Mol Microbiol 1994;14:51–60.
36. Di Tommaso A, Saletti G, Pizza M, Rappuoli R, Dougan G, Abrignani S, et al. Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. Infect Immun 1996;64:974–9.
37. Yamamoto S, Takeda Y, Yamamoto M, Kurazono H, Imaoka K, Yamamoto M, et al. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrhoeagenicity but retain adjuvanticity. J Exp Med 1997;185:1203–10.
38. Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Yamamoto M, Fujihashi K, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci USA 1997;94:5267–72.
39. Lencer WI, Hirst TR, Holmes RK. Membrane traffic and the cellular uptake of cholera toxin. Biochim Biophys Acta 1999; 1450:177–90.
40. Bourguignon P, Bisteau M, Abarca J, Veenstra S, Hermand P, Henderickx V, et al. Reactogenicity in the nose and the brain of enterotoxins administered intranasally to mice. In: Proceedings of the Cold Spring Harbor Conference on Molecular Approaches to Vaccine Design; 1999 Dec 2–5. p. 23.
41. van Ginkel FW, Jackson, RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J Immunol 2000;165:4778–82.
42. Koga T, McGhee JR, Kato H, Kato R, Kiyono H, Fujihashi K. Evidence for early aging in the mucosal immune system. J Immunol 2000;165:5352–9.
43. Czerkinsky C, Anjuere F, McGhee JR, George-Chandy A, Holmgren J, Kieny MP, et al. Mucosal immunity and tolerance: relevance to vaccine development. Immunol Rev 1999;170:197–222.
44. Elson CO, Ealding W. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. J Immunol 1984;133:2892–7.
45. Kato H, Fujihashi K, Kato R, McGhee JR. Prior oral tolerization abrogates cholera toxin-induced IgA responses in gastrointestinal tract. J Immunol 2001;166:3114–21.
46. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 1994;264:703–7.
47. Banks TA, Rouse BT, Kerley MK, Blair PJ, Godfrey VL, Kuklin NA, et al. Lymphotoxin-α-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. J Immunol 1995;155:1685–93.
48. Alimzhanov MB, Kuprash DV, Kosco VM, Luz A, Turetskaya RL, Tarakhovsky A, et al. Abnormal development of secondary lymphoid tissues in lymphotoxin β-deficient mice. Proc Natl Acad Sci USA 1997;94:9302–7.
49. Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA. Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. Immunity 1997;6:491–500.
50. Koni PA, Flavell RA. A role for tumor necrosis factor receptor type 1 in gut-associated lymphoid tissue development: genetic evidence of synergism with lymphotoxin β. J Exp Med 1998; 187:1977–83.
51. Futterer A, Mink K, Luz A, Kosco VM, Pfeffer K. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity 1998;9:59–70.
52. Rennert PD, Browning JL, Mebius R, Mackay F, Hochman PS. Surface lymphotoxin α/β complex is required for the development of peripheral lymphoid organs. J Exp Med 1996;184:1999–2006.
53. Rennert PD, James D, Mackay F, Browning JL, Hochman PS. Lymph node genesis is induced by signaling through the lymphotoxin β receptor. Immunity 1998;9:71–9.
54. Rennert PD, Browning JL, Hochman PS. Selective disruption of lymphotoxin ligands reveals a novel set of mucosal lymph nodes and unique effects on lymph node cellular organization. Intern Immunol 1997;9:1627–39.
55. Fujihashi K, Dohi T, Rennert PD, Koga T, Kiyono H, McGhee JR. Peyer's patches are required for oral tolerance to proteins. Proc Natl Acad Sci USA 2001;98:3310–5.
56. Shroff KE, Meslin K, Cebra JJ. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. Infect Immun 1995;63 3904–13.
57. Wannemuehler MJ, Kiyono H, Babb JL, Michalek SM, McGhee JR. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. J Immunol 1982;129:959–65.
58. Kramer DR, Cebra JJ. Early appearance of "natural" mucosal IgA responses and germinal centers in suckling mice developing in the absence of maternal antibodies. J Immunol 1995;154: 2051–62.
59. Coffman RL, Lebman DA, Shrader B. Transforming growth factor □ specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J Exp Med 1989; 170:1039–44.
60. Sonoda E, Matsumoto R, Hitoshi Y, Ishii T, Sugimoto M, Araki S, et al. Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. J Exp Med 1989;170:1415–20.
61. Mowat AM, Steel M, Leishman AJ, Garside P. Normal induction of oral tolerance in the absence of a functional IL-12-dependent IFN-γ signaling pathway. J Immunol 1999;163:4728–36.
62. Gonnella PA, Chen Y, Inobe J, Komagata Y, Quartulli M, Weiner HL. *In situ* immune response in gut-associated lymphoid tissue (GALT) following oral antigen in TCR-transgenic mice. J Immunol 1998;160:4708–18.
63. Yamamoto M, Rennert PD, McGhee JR, Kweon M-N, Yamamoto S, Dohi T, et al. An alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the GI tract. J Immunol 2000;164:5184–91.
64. Viney JL, Mowat AM, O'Malley JM, Williamson E, Fanger NA. Expanding dendritic cells in vivo enhances the induction of oral tolerance. J Immunol 1998;160:5815–25.