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Perspectives on Mucosal Vaccines: Is Mucosal Tolerance a Barrier?1

Jiri Mestecky,2†§ Michael W. Russell,‡ and Charles O. Elson∗†

Mucosal administration of Ags induces specific Abs in external secretions and systemic unresponsiveness termed oral or mucosal tolerance. The dominant response depends on the species studied, the nature, dose, frequency, route of Ag application, and the use of adjuvants. The temporal sequence of Ag exposure determines the quality of the ensuing immune response; although initial mucosal Ag exposure results in systemic T cell hyporesponsiveness, pre-existing systemic responses are refractory to the tolerizing effects of mucosal Ag encounter. Mucosal and systemic humoral responses may be induced concomitantly with diminished systemic T cell responses, thereby permitting Ab-mediated containment of mucosal Ags without stimulation of the systemic immune compartment. B cell Ig isotype switching and differentiation toward IgA production share common regulatory mechanisms with the suppression of T cells. Optimization of mucosal vaccination strategies has the potential for enhancing protective immune responses and suppressing systemic responses to autoantigens desirable for the treatment of autoimmune diseases. The Journal of Immunology, 2007, 179: 5633–5638.

One of the major functional aspects of the mucosal immune system can be seen as limiting the access of environmental Ags such as food and airborne materials as well as commensal microbes to the systemic immune compartment and, hence, reducing the magnitude of systemic immune responses to such frequently encountered Ags. In view of the enormous antigenic challenge encountered on a daily basis, particularly in the gastrointestinal tract, the survival advantage of effectively controlling Ag uptake and regulating the ensuing immune responses is clear.

The induction of specific Abs at both the site of Ag stimulation and at remote mucosal tissues has been well documented (for reviews see Refs. 1–4). Because such Abs confer protection against mucosal infectious agents or limit the uptake of Ags from mucosal surfaces, extensive studies based on the exploitation of this principle have been conducted on the design of vaccines given by different mucosal routes (1, 2, 4–6). However, despite its physiological and pharmacological attractiveness, there have been few concerted efforts to develop mucosal vaccines. Such vaccines offer certain advantages, including the stimulation of humoral responses at the site of entry of most infectious agents, the simplicity of administration without the need for sterile needles and syringes, and the potential for prompt mass immunization. However, these advantages are sometimes questioned on important immunologic grounds (4, 7). Due to the relatively low rates of absorption of Ags from mucosal surfaces and their degradation by proteolytic enzymes, large doses of Ags may be required to induce the desired immune responses. This further prompts a frequently asked question concerning the possible induction of oral (mucosal) tolerance by mucosal vaccines: can extended and repeated mucosal application of Ags decrease systemic immune responses and is this mucosal tolerance of significant importance in the development and application of mucosal vaccines? In this brief review, we have attempted to critically analyze current thoughts concerning this topic with emphasis on its impact for the future of mucosal vaccine development.

Basic observations

Repeated oral ingestion of large doses of Ags or haptens conjugated to suitable carriers results in decreased or totally abrogated responsiveness to a subsequent systemic immunization with the same Ag (for reviews see Refs. 8 and 9). Such oral tolerance has been extensively studied in animal models, including mice, rats, and guinea pigs; other species appear to be less prone to the development of systemic unresponsiveness. The target of oral tolerance is mainly the T cell compartment and is revealed by altered delayed-type hypersensitivity (DTH)3 reactions, diminished in vitro T cell proliferation, the induction of various populations of regulatory T cells, and the production of suppressive cytokines (8, 9). Multiple mechanisms have been observed, including deletion and/or anergy of Ag-specific T cells and the

1 Address correspondence and reprint requests to Dr. Jiri Mestecky, University of Alabama at Birmingham, Department of Microbiology, Box 1, 845 19th Street South, Birmingham, AL 35294-2170. E-mail address: mestecky@uab.edu

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3 Abbreviations used in this paper: DTH, delayed-type hypersensitivity; CT, cholera toxin; CTB, cholera toxin B; KLH, keyhole limpet hemocyanin; LT, labile toxin; pIgA, polymeric IgA.

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induction of T regulatory cells, and these mechanisms are not mutually exclusive (8–11). In most reports, potential suppression of Ab responses was not extensively studied. An exception may be the apparent suppression of IgE responses to environmental allergens. The induction of secretory IgA or IgG Abs that would successfully compete with IgE Abs for a given allergen or the selective suppression of IgE Ab responses are at the heart of these efforts. Marked differences in the efficacy of oral immunotherapy may be ascribed to the animal species and to the type and purity of allergens, the Ag delivery or adjuvant systems, and the doses used (9, 12).

The ability to prevent or even to reverse experimental T cell-mediated autoimmune diseases such as experimental allergic encephalomyelitis, collagen type II-induced arthritis, or autoimmune diabetes in animal models (4, 8, 9) has led to renewed interest in applying the principles of oral tolerance to the therapeutic treatment of autoimmune diseases (e.g., multiple sclerosis and rheumatoid arthritis) in humans. As explained below, these efforts have not been encouraging (9). This limited effectiveness in humans raises questions concerning the reasons for such a remarkable difference in clinical outcomes compared with results from the animal models. Although the species, age, and gender under investigation as well as the type and dose of Ag, the frequency of exposure, the use of adjuvants and Ag delivery systems, and other parameters may greatly influence the outcome, we propose that prior exposure to the particular Ag plays a decisive role; i.e., once an immune response is elicited that mode of response predominates whenever the same Ag is encountered in the future.

Does mucosal tolerance exist in humans?

The number of studies addressing this point is surprisingly limited despite its enormous medical importance (8–10). Lowney (13, 14) observed the reduced incidence and intensity of cutaneous sensitization in approximately one-half of humans first given an oral application of 2,4-dinitrobenzene in acetone on the oral mucosa.

It is difficult to identify an experimental Ag never previously encountered in humans and to which, therefore, the population has no pre-existing immunity. Such an Ag must also be suitable both for mucosal immunization and for subsequent systemic challenge to test the induction of systemic unresponsiveness. With these limitations in mind, we (15, 16), and others (17, 18) selected keyhole limpet hemocyanin (KLH) because of its high immunogenicity as revealed by both humoral and cell-mediated responses to systemic immunization with small doses. Extended ingestion or intranasal inhalation of KLH by volunteers lacking pre-existing immunity followed by systemic challenge resulted in an unexpected outcome: decreased cell-mediated immunity manifested by diminished DTH reactions and T cell proliferation in vitro but priming for B cell responses. Mucosally immunized volunteers responded to the subsequent systemic KLH injection by higher titers of systemic and secretory Abs than those who received only systemic injection of KLH. Thus, the initial mucosal immunization with high doses of a glycoprotein Ag did not suppress the humoral arm of the immune response. This finding has important implications for mucosally delivered vaccines. Furthermore, extensive studies of humoral and cellular immune responses to food Ags (19–22) clearly demonstrate that humoral immune responses to important components such as bovine γ-globulin, which is consumed in the average American diet at doses of 500 mg per day (~180g/year), persist throughout childhood, adolescence, and early adulthood, with some decrease in later life. Importantly, the levels of Abs to food Ags in sera and external secretions display considerable mutual independence, indicating marked differences in maturation and regulation of both the magnitude and the Ig isotype of the response within the systemic and mucosal compartments (19).

It is important to understand the context in which the mucosal immune system operates. In addition to the large quantities of food Ags ingested daily, the intestine is colonized by a complex microbiota, the members of which are just beginning to be identified (23). The total numbers are estimated at 100 trillion organisms per person, which represents 10-fold more microbial cells than human cells present in the body (24). This is truly an enormous challenge of bacterial Ag and adjuvants for the mucosal immune system, and it is present for the lifetime of the host. The mechanisms by which most of us live in peace with this microbiota are just beginning to be understood. It is clear that the host is responding to this microbial challenge, as witnessed by the large numbers of lymphoid cells present in the intestine and the 3–5 g of IgA produced per day in the normal human (25). This complex microbial ecosystem, our “other self”, is in dynamic communication with the intestinal epithelium and with the innate and adaptive lymphoid cells present in the intestine (26). This is the context into which a mucosal vaccine or tolerogen is being introduced. Much of the difficulty experienced at inducing mucosal immunity or tolerance at will to a given Ag is likely due to our relative ignorance of this microbiota and the resulting host-microbial interactions that are occurring continuously at the mucosal surface.

Despite the large cellular and humoral response to the microbiota, it is commonly thought that the host is immunologically tolerant to these bacteria (27). Against this idea is the active adaptive immune response to the microbiota that is present in mice; this response is compartmented to the intestine, i.e., there is abundant IgA to microbial Ags in the intestine but no serum IgG Abs or systemic T cell responses to these same Ags (28–30). A recent study found no evidence of immunologic tolerance (anergy, deletion, or regulation) of the murine immune response to a set of 20 recombinant microbiota protein Ags; instead, the systemic T cells remain naive to these Ags (30). The intestine also contains IL-10-producing CD4+ T cells reactive to the Ags of the commensal microbiota (31). The mucosal immune response to these Ags appears to be tightly regulated, and details about how regulatory cells are induced and maintained in the mucosa are just emerging (32, 33). Indeed, the phenomenon of mucosal tolerance to protein Ags delivered into the intestine may be related to and be dependent upon the regulation of the immune response to the microbiota. The Ag being delivered to a mucosal surface is, after all, encountered along with the endogenous microbes. There is much less known about the normal human mucosal immune response to the microbiota. Although humans produce IgA Abs to commensal bacteria, such responses do not appear to be confined to the intestine as tightly as in mice as shown by the presence of serum IgG Abs to Ags of the commensals.

Certainly, strategies for the development of effective mucosal vaccines need to consider the large and continuous response to the Ags of the microbiota. A given vaccine Ag is in essence in competition for the attention of the mucosal immune system.
with the many thousands of these microbial Ags that occur in the gut before exposure to the vaccine Ag and will continue long after it has disappeared. The delivery of vaccine Ags by microbes has been tried in various forms. In some systems, commensal bacteria themselves are used to deliver the Ag into the intestine. If there is already an endogenous regulation to the commensal vector, it is likely to be transferred to the vaccine Ag as well. Attenuated pathogens such as Salmonella have been used to deliver vaccine Ags. Such vectors do induce immune responses to vaccine Ags in the short term, but the response to the neoantigen is overwhelmed by the response to the more immunodominant Ags of the Salmonella itself.

In contrast to enhanced or essentially unaltered humoral responses, the administration of Ags to human gastrointestinal or respiratory tracts induces a profound decrease in cell-mediated immunity as manifested by diminished DTH, T cell proliferation, and secretion of cytokines with suppressor activity (15–18). Although most if not all of the currently available human vaccines generate their protective effects through the induction of specific Abs, it is not known whether mucosal exposure to large doses of inactivated microorganisms (viruses, bacteria, or isolated Ags) inhibits the subsequent induction of CTL by either systemic immunization or infection. However, if that is the case, the efficacy of vaccines whose protective function at least partially depends on CTL activity might be compromised. To investigate this, Ilan (34) induced tolerance to adenoviral proteins by feeding them to experimental animals, which then displayed markedly reduced anti-adenoviral humoral as well as cellular immunity as evaluated by increased TGβ, IL-4, and IL-10 but decreased IFN-γ production by lymphocytes upon exposure to adenoviral Ags in vitro. This finding is of considerable importance for gene therapy using adenoviruses as vectors; extended viral survival due to tolerance to the virus and, therefore, longer expression of the desired gene product encoded in a recombinant adenoviral vector would be of great benefit for both gene therapy and viral vector-based vaccination. Conversely, induction of tolerance to a virus as a vaccine might be an undesirable outcome. It is possible that vaccines based on live vectors involving commensal (e.g., Escherichia coli and Lactobacillus) or pathogenic (e.g., Salmonella) bacteria would also show differential vector survival in the gastrointestinal tract as a result of differences in the immune responses to these two types of bacteria (29).

The role of adjuvants in immunity or tolerance

The role of immunomodulating adjuvants is extremely important in this context. Among these, the most intensively researched are the heat-labile enterotoxins of Gram-negative enterobacteria, such as cholera toxin (CT) and the closely related type I labile toxin (LT), LT-I, of E. coli plus the type II toxins LT-IIa and LT-IIb also from E. coli. The mucosal adjuvant properties of CT were first demonstrated by its ability to prevent and even reverse the development of oral tolerance (35), and a voluminous literature has since developed demonstrating the effectiveness of CT or LT-I as mucosal adjuvants when co-administered with a large variety of Ags delivered orally (intragastrically) or intranasally (36). The precise mechanism of action of these adjuvants has not been completely elucidated, and controversies exist over the requirements for and roles of the A and B subunits of these toxins (37). Factors involved in the diverse findings include the route of administration, the nature and properties of the vaccine Ag, contamination of the adjuvant with endotoxin or, in the case of the B subunit, with holo toxin, and also the species of animal used. In addition, the B subunits of enterotoxins can serve as coupled delivery agents for vaccines, but the results differ from those obtained when enterotoxins are used as admixed adjuvants. The manner of coupling, the nature of the coupled Ag, the route of administration, and the species affect the outcome. Ags chemically conjugated to cholera toxin B (CTB) induce mucosal and systemic Ab responses to the coupled Ag when given intragastrically or intranasally in mice, but coadministration of a small adjuvant dose of intact CT may be necessary especially in rats (38–40). In the absence of holo toxin, oral administration of Ags chemically conjugated to recombinant CTB induces profound tolerance in mice (41). Even previously established systemic immune responses were suppressed, and T cell-mediated autoimmune conditions such as experimental autoimmune encephalomyelitis could be reversed (42). Regulatory T cells (Treg cells, both Foxp3+) were induced in mice orally immunized with OVA (which readily induces tolerance) conjugated to CTB (43).

In contrast, genetically coupled recombinant chimeric proteins of the form Ag-A2/B3, in which Ag is fused to the A2 subunit of the enterotoxin and assembled with B subunits, are immunogenic in mice in the absence of an intact holo toxin adjuvant and no evidence of tolerance induction has been found (44–46). Although the mechanisms by which these different Ag-enterotoxin constructs interact with APC to induce tolerance or immunity are not understood, clearly there are differences between the molecular forms that may be amenable to exploitation for the elicitation of diverse responses. However, few trials have been conducted in humans. Oral administration of a peptide derived from human heat-shock protein-60 fused to CTB was found to prevent relapses of uveitis in Behcet’s disease (47). Human vaccine trials have commenced using non-toxic mutants of LT-I with Helicobacter pylori or an intranasal influenza vaccine (48, 49).

Can an existing immune response to a foreign Ag or an autoantigen be suppressed by mucosal immunization

In animal models of type IV DTH to a hapten or of autoimmune diseases such as experimental allergic encephalomyelitis, collagen type II-induced arthritis, and autoimmune diabetes mellitus, prior mucosal exposure to the relevant Ag by the oral or intranasal route diminishes or prevents the development of a reaction or disease otherwise induced by systemic immunization (4, 8, 9, 41–43, 50). Based on these findings, the important question was raised as to whether this desirable effect could be extended to the therapeutic exploitation of mucosal tolerance in the treatment of pre-existing autoimmunity or DTH. Early empirical studies on the suppression of DTH reactions to environmental Ags, such as poison ivy or poison oak, provided conflicting but mostly discouraging results; feeding fresh, dried or extracted leaves of these plants proved of no benefit in most trials (51, 52). It has been demonstrated in both animals and humans that pre-existing systemic immunity effectively precludes the induction of mucosal tolerance. Chase (53) convincingly demonstrated and explicitly stated in his now rarely cited landmark paper that animals first systemically sensitized by a hapten (2,4-dinitrobenzene) are totally refractory to the beneficial effect of subsequent oral immunotherapy. Similarly, animals with well-established, progressive autoimmune disease
(experimental allergic encephalomyelitis or collagen type II-induced arthritis) induced by initial systemic immunization respond minimally if at all to oral or intranasal immunotherapy (4). Initial systemic immunization of human volunteers with KLH followed by extended ingestion of large doses of the same Ag failed to generate tolerance (54). In this study, the measurement of Ag-driven T cell proliferation, KLH-specific serum and salivary Ab levels, and cutaneous DTH responses failed to reveal significant differences between subjects fed KLH or OVA as a negative control Ag. Thus, systemically induced primary responses in humans were not attenuated by subsequent oral ingestion. However, whether these data can be extended to other neo-Ags or vaccines given over a much broader range of doses is unknown. This point is important for immunization with mucosally administered vaccines as well as the potential treatment of autoimmune diseases. In the first case, it is unlikely that mucosal immunization of an individual with even a low pre-existing level of systemic immunity would induce T cell-mediated systemic unresponsiveness. In the second situation, it is becoming apparent that the limited success in the treatment of existing autoimmune diseases by mucosal immunization with the autoantigen (8, 9, 55, 56) can be explained by the failure to suppress a pre-existing systemic response.

Functional complementarity of mucosal Ab responses and mucosal tolerance: common regulatory mechanisms

The IgA isotype switching of B cells and their differentiation into specific IgA Ab-producing plasma cells (57–59) and systemic T cell hyporesponsiveness, both of which are induced by mucosal exposure to Ags (8, 9, 60), display complementary functional effects and may share some common regulatory pathways. Although specific Abs of any major Ig isotype in secretions prevent the uptake of inert Ags from mucosal surfaces and restrict the penetration of microorganisms into the systemic compartment, Abs of the IgA isotype display unique functional advantages, particularly upon Ag encounter in mucosal tissues. In addition to their pronounced resistance to intestinal proteolytic enzymes and the beneficial effect of multivalency (four or eight Ag binding sites per dimer or tetramer, respectively), specific IgA Abs exhibit strong anti-inflammatory activity manifested by the inhibition of complement activation and of the activation of inflammatory cell types (3, 5, 61–63). This in turn reduces potential tissue damage, breakdown of the mucosal barrier, and indiscriminate uptake of bystander Ags (63). In addition, the integral involvement of mucosal epithelial cells in receptor-mediated transcytosis of locally produced polymeric IgA (pIgA) facilitates the clearance of Ags complexed with a specific IgA Ab (64).

Despite this IgA-mediated prevention of mucosal uptake, minute amounts of undigested Ags (e.g., bovine γ-globulin from milk) can be detected in the circulation, usually in the form of IgA-containing immune complexes (65). In addition, the parallel induction of mucosal Ab responses and the suppression of systemic cell-mediated reactions reduce stimulation of the immune system. Importantly, all of these pathways, including isotype switching to IgA and terminal differentiation of IgA plasma cells, epithelial transcytosis of pIgA and the induction of mucosal tolerance are regulated by common cytokine networks (Fig. 1). Subsets of CD4⁺CD25⁺ or CD4⁺CD25⁻ regulatory T cells secrete cytokines, including TGFβ, IL-4, and IL-10, that participate in surface IgM⁺ to surface IgA⁺ isotype B cell switching, IgA B cell differentiation (TGFβ and IL-10), epithelial cell expression of the pIgA receptor and transcytosis (IL-4), and the suppression of cell-mediated immunity (TGFβ, IL-4, and IL-10) (8, 9, 57–59, 64, 66–68). Although murine B1 cells can differentiate into IgA-producing cells that secrete Abs specific for intestinal microbes independently of T cells (28), this may not be the case in humans because B1 cells are not detectable in the intestinal lamina propria (59). We speculate that the induction of systemic T cells and the stimulation of IgA responses may result concomitantly from the induction of these “inhibitory” cytokine networks.

Unresolved questions

Although the parallel induction of both mucosal Ab responses and mucosal tolerance after Ag ingestion has been demonstrated first in mice (60) and then in humans (15, 16), exploitation of the potential of this phenomenon will require further investigation. Ags used to date in humans have included only contact allergens (13, 14) and KLH (15–18). It is well known that mucosal immunization with biologically relevant complex Ags such as inactivated viruses, bacteria, and their products induces mucosal as well as systemic Ab responses (1, 2, 4), but its impact on cell-mediated immunity, particularly the induction of systemic and mucosal CTL responses, has not been adequately investigated. An important consideration in this context is that mucosal immunization of immunologically naive subjects not previously exposed to HIV with a potential HIV vaccine might have the undesirable effect of diminishing cell-mediated, CTL-dependent immunity. This problem may not apply to other mucosal vaccines (e.g., poliovirus, influenza virus) that, like other currently used vaccines irrespective of the immunization route, achieve their protective effects through
the induction of specific Abs rather than CTL. Because of pre-existing systemic immunity induced by prior infection or systemic immunization, the likelihood of inducing mucosal tolerance by mucosally administered vaccines is small. Furthermore the choice and order of immunization routes (e.g., systemic priming followed by mucosal boosting), Ag dose, delivery system, and use of mucosal adjuvants or immunomodulatory cytokines may skew the magnitude and quality of the immune response toward the desired outcome (1, 2, 4).

Finally, different mucosal sites of initial Ag exposure, e.g., oral cavity, intestinal tract, nasal mucosa and lungs, or female genital tract, may not be equally effective in the induction of mucosal and systemic responses or tolerance. Moreover, tolerance induction by vaginal application of Ag may be subject to hormonal regulation; in mice, tolerance can be induced vaginally during estrus but not during diestrus (69). It is likely that the physiological microenvironment of various mucosal surfaces and the presence or absence of inductive sites with characteristic resident cell populations, including macrophages, dendritic cells, epithelial cells, B cells, and T cells (4, 8, 9, 66–68, 70–73), will influence the balance of the ensuing immune response in a physiologically determined direction which may be further exploited in mucosal vaccination.

Disclosures

The authors have no financial conflict of interest.

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