



Review

Regulation of Immunological Mucosal Tolerance

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Abstract. Mucosal tolerance is an immunological phenomenon specific to mucosal surfaces as found in the lungs and gastrointestinal tract. It results in the suppression of immune responses to inhaled or ingested antigens and prevents the body from unwanted and unnecessary immunological responses to harmless molecules, such as grass-pollen or food constituents. This imposes the difficult task for the immune system of keeping a balance between reacting and non-reacting, and disturbances of this balance result in allergies and, possibly, autoimmunity, as well as opportunistic infections and even an escape from tumor surveillance. Understanding the mechanisms that underlie mucosal tolerance is, therefore, important from different viewpoints. Maintenance or (re)induction of mucosal tolerance to, e.g., food proteins, airborne allergens or autoantigens is desirable to prevent or cure allergies and autoimmune diseases. However, induction of mucosal tolerance is an unwanted phenomenon in mucosal vaccination and in the case of mucosal tumors.

Key words: tolerance; mucosa; nasal; lymph node.

Immunological Tolerance as a Protective Mechanism

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Antigens and Dose of Antigen that Can Induce Mucosal Tolerance

There is a wide variety of antigens that are capable of inducing mucosal tolerance, such as protein antigens, contact sensitizers, inactivated bacteria and viruses, peptides and autoantigens. Immunogenic peptides are

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usually very effective, but also epitopes distinct from immunogenic ones have been reported to mediate immune suppression. The degree of tolerance acquired with a certain peptide is improved by increasing the affinity of the peptide to its major histocompatibility complex (MHC) restriction element.

The dose of mucosally applied antigen is another factor that modulates the effectiveness and mechanism of tolerance. Very low doses of antigen might rather prime immune responses than induce tolerance²⁰. In general, orally induced tolerance is divided in low-dose and high-dose tolerance, resulting in either a state of tolerance maintained by a regulatory population or the induction of anergy or deletion of antigen-specific T cells. These three mechanisms of mucosal tolerance will be discussed below. Reports on nasally or aerosol-induced tolerance do not mention the involvement of anergy or clonal deletion. This is either related to the putative intrinsic differences in regulation between oral and airway tolerance or simply a consequence of the limited amount of antigen that can be administered via the nose. Moreover, the uptake of large proteins by the nasal epithelium occurs at a very low rate. Notwithstanding the generally accepted division in high- and low-dose tolerance, this division is probably not strict, and high- and low-dose tolerance are not mutually exclusive. Moreover, the particular antigen or immune response involved can influence dose dependency, since, for example, oral doses of collagen of 1 mg or more do not suppress arthritis¹⁷, while equal doses of insulin or myelin basic protein (MBP) readily suppress diabetes or experimental autoimmune encephalomyelitis (EAE)³⁵.

Immune Responses that are Affected by Mucosal Tolerance

The effect of mucosal tolerance has been intensively studied on T helper (Th)1-mediated immune responses, such as delayed-type hypersensitivity (DTH) and EAE. In these models, the occurrence of mucosal tolerance is often associated with up-regulation of Th2 responses^{3, 26}. Strikingly, one of the responses most readily affected by mucosal tolerance induction is the typically Th2-regulated IgE response by oral as well as nasal administration of antigen^{20, 29}. Moreover, high-dose oral tolerance can be successfully induced in IL-4^{-/-} mice⁷. Overall, these data rather conflict with the theory that mucosal tolerance is mediated by Th2 cells. When mice were treated systemically or locally with anti-IL-4 or anti-IL-10 antibodies before intranasal tolerance induction the extent of tolerance as measured by suppression

of DTH was not affected. Then up-regulation of Th2 responses observed after intranasal tolerance is, therefore, rather an additional than a necessary reaction³⁴.

Besides to suppression of Th cells, mucosal tolerance can also affect CD8⁺ cytotoxic T lymphocytes (CTLs)¹³, implying that the MHC class I-restricted pathway can also be subjected to mucosal tolerance. Inhibition of CD8⁺ cells can be achieved by a CD4⁺ regulatory population, as has been demonstrated by the impairment of oral tolerance induction for a CD8⁺ T cell-mediated contact hypersensitivity response in CD4-depleted mice. Alternatively, since CD8⁺ cells might need help from CD4⁺ cells via IL-2, suppression of CD8⁺ cells can occur indirectly via suppression of T helper cells.

To date, a considerable number of reports have focused on clinically relevant models to study the possibilities of employing mucosal tolerance to cure or prevent autoimmune diseases and allergies. Various data exist on the suppression of experimental autoimmune diseases, including EAE¹⁰, collagen- and adjuvant-induced arthritis²¹, myasthenia gravis and diabetes^{16, 35}, by oral administration of autoantigens. Aerosol or intranasal administration of autoantigens has been shown to be at least equally effective²⁶. In addition, efficient inhibition of allergic responses has been achieved in models for food allergy²⁹, contact hypersensitivity³⁰ and house dust mite allergy¹². Moreover, mucosal administration of alloantigens might provide an approach to suppress allograft rejection²⁴.

Although most studies aim at induction of tolerance before immune priming or the onset of disease, it is clinically of interest to induce tolerance after immune activation. Tolerance dominates when the tolerogenic stimulus precedes immune activation. If the tolerogenic signal is given within a relatively short time-span after immunization, suppression can still be detected, and an increased frequency of administration enhances tolerance. Moreover, relapsing EAE can be suppressed after the onset of disease by oral administration of MBP or myelin. Although studies carried out in patients suffering from multiple sclerosis or rheumatoid arthritis^{27, 31} suggest the possibility to interfere in ongoing immune responses by the feeding of autoantigens, mucosal tolerance induction in the treatment of autoimmune diseases should still be considered with care. The reasons for this are unclear, but may be related to changes in the established memory T cell pool⁴.

Induction of Mucosal Tolerance

Lymphoid tissues lying along the mucosa are collectively termed mucosa associated lymphoid tissue

(MALT) but the specific role of MALT in the induction of mucosal tolerance is unclear. It has been shown that rats without Peyer's patches (PP) can still become tolerant after antigen feeding. On the other hand, the PP have been described as induction sites for regulatory T cells and transforming growth factor β (TGF- β) production, associated with tolerance²³.

The lymphoid tissue draining the mucosa is considered as specialized lymphoid tissue with properties distinct from those of the peripheral lymph nodes. Usually, it contains a larger B cell compartment and the CD4/CD8 ratio is smaller than in peripheral lymph nodes. MALT is often regarded as a Th2-type environment, due to the preferential production of Th2 cytokines⁶. In a mouse model we have shown that the superficial cervical and internal jugular lymph nodes, which drain the nasal mucosa, are instrumental for the induction of tolerance. Removal of these lymph nodes abrogates nasal tolerance induction, which can be restored by transplantation of superficial cervical lymph nodes, but not of peripheral lymph nodes³³. The results indicate that in the lymph nodes which directly drain the nasal mucosa a unique microenvironment is created, which favors the induction of tolerance.

In what way are these lymph nodes different? A characteristic property of mucosa-draining lymph nodes is the expression of distinct adhesion molecules. The expression of the mucosal addressin MAdCAM-1 on the high endothelial venules of mucosal lymphoid organs, such as PP and mesenteric, and cervical lymph nodes, ensures the immigration of $\alpha 4\beta 7^+$ lymphocytes into mucosal lymphoid organs. So far no data exist on a specific role either for these lymphocytes or for MAdCAM-1 in the process of mucosal tolerance induction. In addition to draining lymph nodes as important sites for induction of tolerance, the systemic distribution of antigen, e.g. to the spleen, was found to contribute significantly to tolerance induction⁸.

Another specific characteristic intrinsic to the capacity of tolerance induction may lie in the metabolism of the steroid hormone dehydroepiandrosterone (DHEA). Mucosa-draining lymph nodes have a reduced capacity to convert the prohormone dehydroepiandrosterone-sulphate (DHEAS) into the bioactive steroid hormone DHEA compared with peripheral lymphoid organs⁶. However, administration of DHEA before the induction of intranasal tolerance could not change tolerance induction³². When we determined the effect of DHEA after the induction of intranasally induced tolerance, it was clear that the steroid hormone and some of its derivatives were able to break tolerance when administered at the time of systemic immuniza-

tion. These findings might have implications for the regulation of intranasal tolerance and the use of DHEA.

Uptake of Antigen and Presentation at Mucosal Surfaces

After inhalation or oral uptake, antigen is subjected to enzymatic processes in the lumen of the airway system or the gastrointestinal tract. In the nasal cavity, enzymes with aminopeptidase activity are dominant and are present within the nasal cavity or in the epithelial cells. Enzymatic digestion of large proteins might modulate the induction of intranasal tolerance, since a close negative correlation exists between nasal absorption and molecular weight.

Although in the gastrointestinal tract proteins are rapidly digested into peptide fragments, absorption of intact protein has also been reported, and whether digestion is necessary for the induction of oral tolerance remains elusive.

After passage of the epithelium, antigen is taken up, processed and presented by antigen-presenting cells (APC) including macrophages, dendritic cells, B cells and epithelial cells.

The most vigorous stimulators of T cells are dendritic cells, which can be found in the epithelial layer of both the respiratory tract and the intestine. They resemble Langerhans cells in the skin and are able to take up, process and present antigen to T cells. Moreover, in the PP, APC are present that can rapidly capture ingested antigen and stimulate T cell proliferation. Also, dendritic cells in lymph coming from the intestine can normally activate T cells¹⁵.

Besides antigen presentation by dendritic cells, evidence is accumulating that epithelial cells can function as APC, since they can express MHC class II and activate CD4⁺ T cells. Intestinal epithelial cells may also induce T cell anergy, probably through secretion of TGF- β or by a lack of costimulatory molecules. In contrast, CD8⁺ T cells can be activated by intestinal epithelial cells, probably via the non-classical MHC class I molecule CD1d.

Mechanisms of Tolerance

Three primary mechanisms for mucosal tolerance have been proposed. These are clonal deletion, clonal anergy and active suppression mediated by regulatory T cell populations. Generally, the latter is induced fol-

lowing low-dose antigen application, while deletion and anergy follow upon high-dose feeding.

Clonal deletion involves the physical elimination of cells and is achieved by apoptosis. Studies in TCR-transgenic mice have indicated that, after the oral administration of a high dose of antigen, antigen-specific T cells of both Th1 and Th2 phenotype are peripherally deleted¹. Deletion was preceded by activation and a large percentage of the cells underwent apoptosis in the dome area of PP.

Anergy is characterized by the inability of a T cell to produce its own growth factor IL-2 upon normal stimulation by antigen and APC, resulting in failure to proliferate. This state of unresponsiveness is due to the absence of costimulation or proliferation at the time of the first antigen encounter. Anergy is probably not a permanent state, as the addition of exogenous IL-2 can abolish the unresponsive state *in vitro*.

Anergy usually follows upon high-dose antigen feeding. This might lead to the presence of high doses of antigen in the circulation, a situation that resembles peripheral, i.e. i.v.-induced, tolerance. Alternatively, the presentation of antigen by cells that cannot provide adequate costimulation, e.g. epithelial cells, can induce anergy.

Both Th1 and Th2 cells can be subjected to anergy induction, though not necessarily concomitantly. Moreover, the production of cytokines and some effector functions are not always blocked in anergic T cells. Control of the immune response by anergy induction might, therefore, predominantly come from the prevention of clonal expansion.

The third mechanism by which mucosal tolerance is thought to be regulated is active suppression. Therefore, the development of antigen-specific regulatory T cells that possess the capacity to suppress the development and/or activity of antigen-specific effector populations follows upon mucosal antigen delivery. The specific microenvironment of the mucosa or mucosa-draining lymph nodes as well as the distinct properties of mucosal APC or T cells have been proposed as factors promoting the development of these regulatory T cells. Recently, a critical role for the costimulatory molecule B7.2 on APC has been shown in low-dose, but not high-dose oral tolerance¹⁴.

The occurrence of active suppression can be shown by the active transfer of tolerance through the transfer of lymphocytes from tolerant to naive animals. Rather contradictory data exist about the possible phenotype of the regulatory cells. The immune suppression following upon intranasal tolerance induction is attributed to CD4⁺ T cells^{26, 29}, whereas aerosol treatment favors the

development of CD8⁺ $\gamma\delta$ ⁺ T cells^{9, 17}. Both types of regulatory T cells have been detected in various models for oral tolerance, irrespective of species, antigen or model^{3, 13}. Furthermore, it has been shown that cloned regulatory CD4⁺ T cells from orally tolerized mice are identical to immunoreactive T cells in T cell receptor (TCR) usage, MHC restriction and epitope specificity^{1, 3}.

The Effector Mechanisms of Regulatory T Cells

The concept that regulatory or suppressor T cells play an important role in immune regulation is supported not only by data derived from mucosal tolerance studies, but comes especially from studies on peripheral tolerance and autoimmunity. The ways by which regulatory T cells can exert their function include the existence of anti-idiotypic regulatory cells that specifically recognize TCR of immunogenic T cells, and the secretion of T suppressor factors, which might consist of the soluble TCR α chain. However, in the case of mucosal tolerance, these options have received little attention so far. Instead, studies have focused on the secretion of cytokines that can mediate immune suppression.

The development of regulatory populations after antigen feeding often coincides with an increased capacity to produce Th2 cytokines and TGF- β . Production of the latter has been attributed to a newly termed subpopulation, Th3³. Studies from the laboratory of Weiner have shown that suppression by regulatory T cells in an oral-tolerance model for EAE is predominantly mediated by TGF- β ³¹. Moreover, oral tolerance in an experimental colitis model is also mediated by this cytokine²². However, some studies report a stimulating role for TGF- β in the development activity of Th1 lymphocytes. How these observations relate to the suppression of Th1-mediated diseases by TGF- β remains elusive. Furthermore, suppression of IgE responses by CD8⁺ $\gamma\delta$ TCR⁺ T cells after aerosol tolerance is not associated with increased amounts of TGF- β production¹⁷. Instead, these cells secrete enhanced amounts of IFN- γ . In studies on intranasal tolerance using TCR transgenic mice, it was seen that a stable population of antigen-specific CD4⁺ T cells survived, but remained refractory to antigenic stimulation. No regulatory CD8⁺ T cells were required and no immune deviation based on selective Th1 or Th2 cytokine secretion seemed to be involved²⁸. This is in line with studies in mice deficient in either Th1 or Th2 cells that can still maintain orally induced peripheral nonresponsiveness²⁵.

Besides direct suppression of effector T cells by regulatory T cells, the phenomenon of bystander sup-

pression is sometimes observed. Regulatory T cells that respond to an antigen introduced via the mucosa, release antigen-nonspecific cytokines that will suppress T cells responding to unrelated antigens in the same microenvironment. This was described *in vivo* and *in vitro* in both intranasal and oral tolerance. In the case of oral tolerance, the suppressive cytokine involved appeared to be TGF- β ¹⁹.

It is unclear what happens to potentially immunoreactive T cells that are exposed to the activities of regulatory T cells. The lack of proliferation and/or activation of these cells might indicate the induction of anergy or can be due to deletion.

Modulation of Mucosal Tolerance

The natural modulation of mucosal tolerance occurs through immaturity or aging. Aerosol tolerance cannot be induced in newborn rats until weaning¹¹. Oral tolerance cannot be induced in neonatal rats or mice (24 hours old), instead, oral administration of antigen rather primes immune responses¹⁸. After the first week of life, the capacity to mount tolerance increases gradually, interrupted by a refractory period around the time of weaning. Transfer of adult splenocytes into neonates restores the capacity to mount mucosal tolerance¹¹, suggesting that immaturity of the immune system, rather than aberrant antigen handling by the mucosa, accounts for the failure to induce tolerance in neonates. Also, aging mice (>24 weeks) become less susceptible to oral tolerance induction and rather develop antibody responses upon antigen feeding.

Prevention of mucosal tolerance can be achieved by pharmacological agents, such as cyclophosphamide, 2'-deoxyguanosine and oestradiol, presumably by interfering in the development of regulatory T cells. In addition, prevention of oral tolerance has been observed upon IFN- γ treatment at the time of antigen feeding, whereas administration of rIL-12 in orally tolerized mice at the site of attempted sensitization could reverse the tolerant state⁵.

Conclusion

Mucosal tolerance is an important immunological phenomenon which prevents the body from overreacting to harmless antigens. Its mechanism is not yet completely understood and the various mucosa that are capable of inducing tolerance may use different mechanisms, depending on the interactions between

local epithelia and draining lymphoid tissue. Although anergy and deletion are prominent ways to achieve tolerance, it can be envisaged that for long-lasting tolerance regulatory T cells capable of immunological memory induction are key players. Their nature and the way they achieve this are still largely unclear.

The immunological specificity of mucosal tolerance makes it a wonderful tool to try to manipulate the immune system when aberrations have occurred such as in allergies and autoimmune disorders. For optimal therapeutic use, however, we will have to know more about the induction mechanisms. Only then will we be able to design ways to overcome the obvious drawback of the system, which, at the moment, makes it virtually impossible to induce tolerance to an antigen against which one is already sensitized.

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