

REVIEW

Nasal tolerance induction as a potential means of immunotherapy for autoimmune diseases: implications for clinical medicine

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Introduction

Antigen-specific therapy of autoimmune diseases has been designed to target T-cell activation [1]. This could be achieved by multiple approaches, as exemplified by studies in experimental autoimmune encephalomyelitis (EAE) and neuritis (EAN). These approaches involve: administration of myelin antigens in soluble form; intravenously (i.v.) at large doses [2–5] or intrathymically (i.t.) [6,7]; intracutaneously (i.c.) [8] or intraperitoneally (i.p.) [9] with incomplete Freund's adjuvant (IFA); i.v. injection of syngenic splenic antigen-presenting cells (APC) that have been pulsed with myelin antigen and chemically cross-linked using ethylcarbodiimide (ECDI) [10–13]; or oral tolerance induction [14–17]. A special form of tolerogenic approach of increasing interest is administration of autoantigen via the nasal route [17–19].

Nasal antigen administration: a highly efficient route of inducing clinically relevant tolerance

In the nasal and upper respiratory tract of humans and animals, there is an intact mucosal immune system. The nasal lymphoid system mainly contains two structures, i.e., nasal associated lymphoid tissue (NALT) and the bronchus associated lymphoid tissue (BALT). NALT in rodents is a bilateral strip of non-encapsulated lymphoid tissue underlying the epithelium on the ventral aspect of the posterior nasal passage [20]. BALT includes lymphoid aggregates that are randomly distributed along the bronchial tract [21]. NALT and perhaps BALT are important structures for induction of either immunity or tolerance. Intranasal immunization has long been used as a route for vaccine

administration. Nasal delivery of a vaccine is more effective in inducing both systemic and generalized mucosal immune responses compared with the oral route [22]. Interestingly, nasal immunization with soluble antigen alone could induce systemic tolerance to the antigen [23]. This property has been used to develop antigen-specific therapy in autoimmune diseases and allergy.

Nasal administration of natural antigens has been shown to suppress a number of experimental autoimmune diseases. We have shown that nasal administration of bovine myelin basic protein (MBP) prevented protracted-relapsing experimental autoimmune encephalomyelitis (EAE) in DA rats [24]. EAE serves as an experimental model for the human disease multiple sclerosis (MS) [25–28]. In our study, rats receiving 600 µg MBP nasally were almost completely protected from EAE. This protection was confirmed at the histological level and associated with decreased levels of MBP-reactive IFN γ secreting TH₁-like spleen cells, decreased serum anti-MBP IgG_{2b} antibody levels and marginally increased anti-MBP IgG₁ antibody levels, indicating that nasal administration of bovine MBP did induce a tolerant state in the DA rats [24]. Similarly, nasal administration of guinea pig (gp)-MBP at a dose as low as 30 µg/rat completely prevented gp-MBP induced active EAE in Lewis rats. Cellular immune responses reflected by antigen-specific T-cell proliferation and IFN γ secretion at the single cell level were suppressed in rats receiving gp-MBP nasally [29]. In both animal models, rats tolerized with MBP also had abrogated MBP-induced IFN γ mRNA expression in draining lymph node mononuclear cells (MNC) compared with rats receiving only PBS nasally. This indicates that nasal tolerance induction is associated with down-regulated TH₁ responses.

Nasal administration of auto-antigens to suppress experimental autoimmune diseases has been shown to be effective in other autoimmune animal models as well. We

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[30] have shown that nasal administration of acetylcholine receptor (AChR) suppressed experimental autoimmune myasthenia gravis (EAMG), which is caused by autoantibodies against AChR of striated muscles. EAMG is an experimental model of myasthenia gravis (MG) in humans. The anti-AChR antibody production in EAMG is T-helper cell dependent [31–33]. A similar report published recently showed that nasal administration of recombinant fragments of the human acetylcholine receptor α -subunit suppressed EAMG [34]. Dick *et al.* [35,36] reported that nasal administration of retinal antigens suppressed retinal antigen-induced experimental autoimmune uveoretinitis (EAU), a T-cell mediated autoimmune disease of the eye. Aerosol insulin has been shown to prevent murine insulin-dependent diabetes, a T-cell mediated autoimmune disease of the β cell islets of the pancreas [37]. Interestingly, nasal administration of multiple autoantigens suppressed multiple auto-immune diseases induced in a single animal, indicating the potential use of this approach in preventing multiple autoimmune diseases by multiple autoantigen-based vaccination [38].

Suppression of autoimmune disease has also been documented after nasal administration of soluble peptides that contained defined T-cell epitopes. We [39] have shown that nasal administration of MBP 68–86 and 87–99, the two encephalitogenic T-cell epitopes in the Lewis rat EAE model, could completely suppress EAE induced with gp-MBP. Nasal administration of peptide 57–81 of the peripheral nervous system myelin protein P2 suppressed experimental autoimmune neuritis (EAN) [40], which is a CD4⁺ T-cell mediated autoimmune disease of the peripheral nervous system, and an experimental model of Guillain-Barré syndrome in humans. Similarly, Metzler and Wraith [41] have shown that nasal administration of an altered immune dominant T-cell epitope of Acl-9 in the H-2^U mouse EAE model, could prevent EAE development and suppress ongoing EAE. In this case, the peptide was modified from the native form so that it had a higher binding affinity to the appropriate MHC. This indicates that peptides can be modified to be more effective to induce tolerance of T-cells. Intranasal administration of the insulin peptide B-(9–23) [42] and glutamate decarboxylase (GAD 65) peptides [43] have been shown to protect non-obese diabetic mice from diabetes. Nasal inhalation of synthetic peptide 184–198, which contains a dominant T-cell epitope of bovine type II collagen (CII), suppressed collagen-induced arthritis (CIA) [44]. Nasal administration of a mycobacterial heat shock protein 60 T-cell epitope in rats suppressed both adjuvant arthritis and non-microbially induced experimental arthritis [45]. Suppression of autoimmune diseases by nasal administration of peptides is likely to depend on the peptide(s) used. Non-dominant peptides did not protect animals from disease [39,46].

Interestingly, many of these studies have shown that the

administration of antigen via the nasal or aerosol route is more effective in inducing T-cell tolerance and suppressing autoimmune diseases in animal models compared with oral antigen administration. The explanation for this could be that in the former case, the tolerogen is placed in immediate contact with the inductive site, the NALT, and is not exposed to the environment of gastric and gastrointestinal proteases before reaching the gut-associated lymphoid tissues (GALT) in the small intestine. Differences in internal properties between NALT and GALT have also not been ruled out. At least, these two structures contain different types of APC [22]. Nasal administration of as little as 30–600 μ g/rat of antigen prior to EAE induction in the Lewis or DA rats induced tolerant states (Table 1). These rats were resistant to subsequent induction of EAE. Nasal administration of 30 μ g/rat of gp-MBP was sufficient to prevent EAE development in the Lewis rats, while a dose of at least 5 mg/rat of gp-MBP was required orally to induce the same extent of tolerance [47]. Our results therefore indicate that antigen administration via the nasal route is highly effective in inducing clinically relevant tolerance.

In studying the feasibility of nasal antigen administration for treatment of human autoimmune diseases, it becomes critical to determine whether nasal antigen administration can suppress an ongoing disease process. Differential treatment effects were achieved with different antigens in different animal models. Since nasal administration of 600 μ g/rat of MBP effectively prevented development of EAE in DA rats, the same, as well as a higher dosage (total 1.6 mg/rat), was used to evaluate the therapeutic effects. Initiation of treatment on day 9 p.i., i.e., when the first clinical signs of EAE appeared, revealed no beneficial effects but rather, a trend of aggravated disease compared with the control rats [48]. In order to investigate whether nasal administration of AChR modulates ongoing EAMG, Lewis rats were treated nasally with AChR, 2 weeks p.i., with AChR and CFA. In this case, 10-fold higher amounts of AChR given nasally (600 μ g/rat) were required to ameliorate the manifestations of EAMG compared with the amounts necessary for prevention of EAMG. No disease aggravation was observed in this model [49]. Suppression of ongoing disease was also observed after giving peptides nasally. Nasal administration of MBP 68–86 + 87–99 could reverse ongoing EAE induced with gp-MBP + FCA, though a higher dosage (about 10-fold higher) was required compared with the dosage needed for the prevention of EAE. MBP 68–86 and 87–99 had synergistic effects [39]. Nasal administration of P2 protein peptides also suppressed ongoing EAN. Again, a higher dosage was required to suppress ongoing disease [50]. Similarly, an altered peptide of Acl-9 could reverse ongoing EAE in H-2^U mice without aggravating disease [41].

Table 1. Differential effects and mechanisms of MBP from various sources and of gp-MBP peptides in inducing tolerance

Exp. no.	Tolerogen	Dosage (µg/rat)	Induction of EAE	Protection of EAE	Proposed mechanisms
1	gp-MBP	30	gp-MBP/CFA/LEW	+ + + +	Regulation
2	gp-MBP	600	gp-MBP/CFA/LEW	+ + + +	Anergy/deletion
3	b-MBP	600	gp-MBP/CFA/LEW	0	0
4	b-MBP	600	rat SCH/IFA/DA	+ + +	Regulation
5	gp-MBP 68–86	600	gp-MBP/CFA/LEW	+ + +	Anergy/deletion
6	gp-MBP 87–99	600	gp-MBP/CFA/LEW	+	0
7	gp-MBP 110–128	600	gp-MBP/CFA/LEW	0	0
8	gp-MBP 68–86 + 87–99	300	gp-MBP/CFA/LEW	+ + + +	Anergy/deletion

0, No protection; +, weak protection; + + +, strong protection; + + + +, complete protection. gp = Guinea pig; b = bovine; SCH = spinal cord homogenate; CFA = complete Freund's adjuvant; IFA = incomplete Freund's adjuvant. This table is based on [24,39,57].

Mechanisms of nasal tolerance induction

The accumulating data thus far indicate that the mechanisms of nasal tolerance induction could be very complicated, and that multiple mechanisms may be involved. One line of evidence indicates that similar to oral tolerance, both T-cell anergy/deletion [47,51] and active suppression [52–56] may be involved in nasal tolerance induction. In the Lewis rat EAE model, we demonstrated that rats pre-treated with a higher dosage of gp-MBP [29] or its immunodominant peptides [39] induced a tolerant state, which was associated with strongly down-regulated antigen-specific T-cell responses. Tolerance could not be adoptively transferred to naive Lewis rats. Similarly, intranasal exposure to a high dose of protein antigen OVA induced immunological tolerance due to T-cell deletion and anergy [57]. These data indicate that T-cells in these tolerized rats were either anergized or being deleted.

However, abundant evidence indicates that the nasal approach is more likely to induce 'regulatory elements' that have been extensively described in other experimental systems [58–60] and in the oral tolerance system [15]. The regulatory elements have two features: first, they could be adoptively transferred to naive animals and protect them from autoimmune disease induction; and second, while the induction of regulatory elements is antigen-specific [14,60], the regulatory elements could non-specifically suppress inflammation in the microenvironment of the target organ. We have shown that nasal administration of bovine MBP could almost completely prevent EAE development in DA rats that was induced with whole syngenic spinal cord homogenate which, besides MBP, contains multiple autoantigens [24]. Metzler and Wraith [41] have shown that nasal administration of minute

amounts of altered MBP peptide of Ac1–9 could almost completely prevent EAE in the H-2^U mice, that was induced with syngenic whole spinal cord. Similarly, nasal administration of P2 protein peptide suppressed EAN that was induced with peripheral myelin [40]. In the Lewis EAE model, splenocytes from low dose gp-MBP tolerized rats could adoptively transfer protection against EAE [29]. Taken together, these data strongly suggest that regulatory elements play a role in nasal tolerance induction and in the suppression of experimental autoimmune diseases. In the DA rat EAE model, we observed that nasal administration of bovine MBP to DA rats with ongoing EAE exacerbated EAE in the acute phase in conjunction with augmented levels of MBP-reactive IFN γ secreting TH₁-like splenocytes. However, TH₁ responses were down-regulated in the later disease phase [48]. This implies that transient activation of TH₁-like cells may precede the induction of regulatory elements *in vivo*. This phenomenon has also been observed in other studies, where transient activation of CD4⁺ T-cells preceded the development of tolerance *in vivo* in mice administered house dust mite (HDM) antigen nasally [61].

The cellular and molecular basis of the regulatory elements acting in nasal tolerance is unknown. A recent study showed that splenocytes from insulin aerosol-treated mice suppressed adoptive transfer of diabetes by T-cells from diabetic to non-diabetic mice. This suppression was shown to be caused by the low numbers of CD8⁺ $\gamma\delta$ T-cells that secrete IL-10 [37,62]. Studies of oral tolerance in the Lewis rat EAE model have shown that the suppressive effect of oral antigen administration could be attributed to TGF- β , which was released by CD8⁺ T-cells upon antigen-specific stimulation [53,54]. Similarly, nasal administration of a µg dose of AChR induced a tolerant state in the Lewis

rat to EAMG, which was associated with high numbers of AChR-reactive TGF- β mRNA expressing cells [63]. Nasal administration of glutamate decarboxylase (GAD65) peptides induced TH₂ responses and prevented murine insulin-dependent diabetes [43]. In EAE, we found that bovine MBP-tolerized DA rats had high numbers of MBP-reactive IL-4 and TGF- β mRNA expressing cells in the spleen and/or spinal cord, and low dose gp-MBP-tolerized Lewis rats had MBP-reactive IL-4 mRNA expressing cells in draining lymph nodes [24]. Our data therefore support a role for IL-4 and/or TGF- β mediated regulatory elements in the induction of nasal tolerance. The phenotype(s) of IL-4 and/or TGF- β producing cells have not yet been completely clarified. Our recent findings in the murine EAMG model indicate that CD4⁺ TH₃ cells may be those producing TGF- β , since splenocytes from AChR-pre-treated wild type and CD8-deficient mice, but not from CD4-deficient mice, suppressed AChR-specific lymphocyte proliferation. This suppression was blocked by antibodies against TGF- β [64].

As noted above, mechanisms behind nasal tolerance induction may vary from antigen to antigen, and both regulatory mechanisms and anergy/deletion of autoreactive T-cells may be involved. Furthermore, for a given antigen like gp-MBP, the mechanisms behind nasal tolerance induction may depend on the dose of tolerogen used, i.e., nasal administration of high doses of tolerogen can result in induction of anergy/deletion of autoreactive T-cells, while a low dose of soluble antigen can lead to peripheral tolerance by a process of active suppression [29].

Taken together, we propose a sequence of mechanisms involved in nasal tolerance induction, as exemplified for the EAE model in Fig. 1. Briefly, for a given myelin antigen, once exposed to nasal and bronchial lymphoid tissue (NALT and BALT) at high concentration, TH-cells specific for this antigen will be deleted or confer anergy [57]. If the antigen is exposed to NALT and BALT at low dose, an active process that involves antigen presentation events will occur. This leads to T-cell expansion and differentiation into antigen-specific regulatory T-cells (T_R) in the NALT and BALT microenvironments. The nature of these T_R-cells is unknown. Phenotypically, they may be TH₂ and/or TH₃ (TGF- β producing) cells. Upon immunization, myelin antigen-specific TH₁ cells are primed in draining lymph nodes. At the same time, T_R-cells also home into the draining lymph nodes, where they meet antigens and are expanded. Once activated, these T_R-cells will produce regulatory cytokines such as IL-4 and/or TGF- β , which will non-specifically suppress TH₁-cell functions. If the functional activity of these regulatory cells is strong enough, no myelin-specific TH₁-cells will be able to get access to the CNS, and the animals will not develop EAE. If T_R-cells are counter-balanced by TH₁-cells, then TH₁-cells will get access to the CNS and evoke inflammation

within the CNS. In this case, T_R-cells can also follow TH₁-cells entering the CNS. Upon encountering antigen, similar processes are evoked as those that took place in the draining lymph nodes. Whether the animals will get disease or remain healthy depends on the functional balance between these two kinds of cells.

Factors that affect nasal tolerance induction

As noted above, many factors can affect the induction of nasal tolerance. These involve the dosage of tolerogen, the property of tolerogen, the genetic background of animals, and the immunogens used to induce disease. As shown in Table 1, gp-MBP was very effective in inducing clinically relevant tolerance in the Lewis rat, while bovine MBP was ineffective in the same model even at higher dosage [29]. This indicates that the antigen structure is critical in inducing nasal tolerance. As shown in Fig. 2, there is only a small difference between the major encephalitogenic epitopes of these protein antigens, though this makes a big difference to their capacity for inducing nasal tolerance. The mechanisms behind nasal tolerance also depend on the property of tolerogens used. For example, bovine MBP prevented EAE in DA rats by regulatory mechanisms, while the same dose of gp-MBP and its peptides induced anergy/deletion of T-cells in Lewis rat EAE. The immunogens and genetic backgrounds of animals are also important factors in nasal tolerance induction. As shown in Table 1, pre-treatment with bovine MBP is tolerogenic to EAE induced with rat spinal cord homogenate + IFA in the DA rat, but not tolerogenic to EAE induced with gp-MBP + CFA in the Lewis rat. Some cytokines like IFN γ could also affect nasal tolerance induction. Co-administration of AChR with IFN γ reversed immune tolerance to AChR and induced EAMG [65].

Complexities of nasal tolerance induction as a therapy for autoimmune disease

A general feature of chronic autoimmune disease is that multiple autoantigens may be involved in the pathogenesis of one organ-specific disorder. During disease progression, intra- and/or intermolecular epitope spreading may occur [66]. Given the complexities of antigens involved in the pathogenesis of autoimmune diseases, therapies based on anergy/deletion of antigen-specific T-cells may not be feasible for clinical use. Instead, antigens or peptides that can induce regulatory elements which will non-specifically suppress inflammation in the target organ may be required. Our studies underline that not all autoantigens or T-cell epitopes that have been identified so far are useful for this purpose when administered by the nasal route. Antigens or

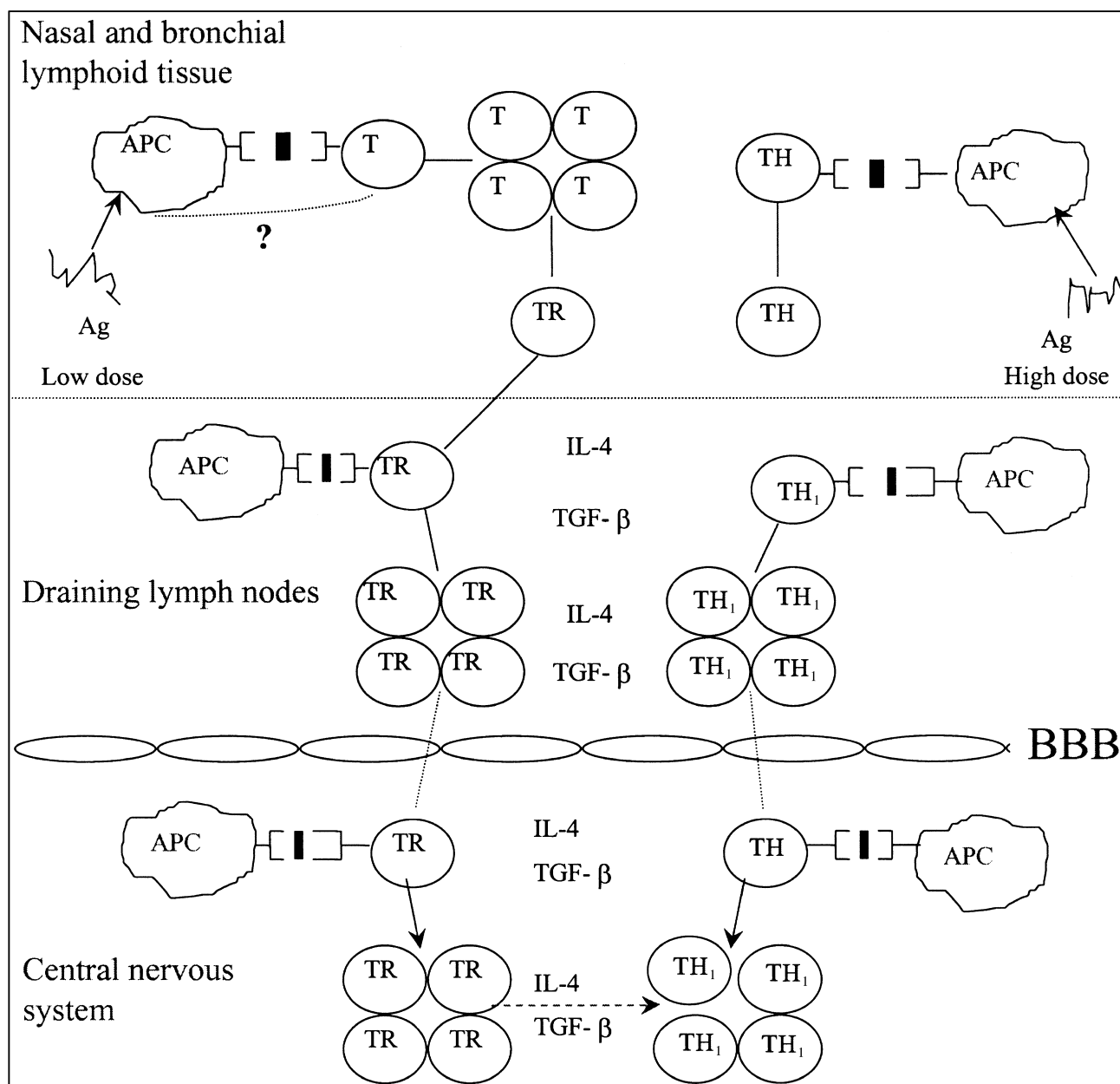


Fig. 1. Mechanisms of nasal tolerance induction in the rat EAE model. For a detailed description see contents of the paper. APC, antigen presenting cell; BBB, blood–brain barrier; TR, regulatory T-cell; TH, helper T-cell.

peptides that can induce regulatory elements [67,68] need to be developed.

Another problem for antigen-specific therapy via the nasal route is that one given antigen is effective in inducing nasal tolerance in one strain but ineffective in another. For example, bovine MBP is effective in inducing nasal tolerance in DA rats, rendering them tolerant to EAE induced with rat spinal cord homogenate + IFA. However, bovine MBP was ineffective in rendering Lewis rats resistant to EAE subsequently induced with gp-MBP + CFA.

Bovine MBP	GSLPQKAQ	GHRP	QDENPVV
Guinea pig MBP	GSLPQKSQ	RT	QDENPVV
Rat MBP	GSLPQKSQ	RT	QDENPVV

Fig. 2. AA sequence of major encephalitogenic epitope region (68–86) from different sources of MBP

Table 2. Effects of nasal administration of MBP and MBP peptides on ongoing EAE

Antigen administered	Dosage (µg/rat)	EAE model	Effect of treatment
b-MBP	600	SCH/IFA/DA	**
b-MBP	1600	SCH/IFA/DA	**/0
gp-MBP 68–86 + 87–99	600	gp-MBP/CFA/LEW	+ +
gp-MBP 68–86 + 87–99	1500	gp-MBP/CFA/LEW	+ + + +

**Worse; 0, not effective; + +, effective suppression; + + + +, complete suppression. b = Bovine; gp = guinea pig; SCH = spinal cord homogenate; IFA = incomplete Freund's adjuvant; CFA = complete Freund's adjuvant. This table is based on [24,39].

Transferred to the human disease situation, a given antigen may be effective in one subpopulation of patients, or one patient, but ineffective in the other.

Ongoing autoimmune disease has been found to be more difficult to suppress (Table 2). Reasons for this may be that: (a) most antigen-specific T-cells have become activated and the frequency of autoantigen-specific T-cells is high; (b) the immune response may have spread to include additional T-cell epitopes; and (c) the effector arms of the immune response, such as inflammatory macrophages, have come into play. Thus, increased amounts of antigens are required to suppress ongoing EAE. Proteins of this magnitude could induce an inflammatory reaction in the nasal mucosa, and possibly in the lungs as well, and thereby preclude their usage for human therapeutic trials. Studies of possible local toxic effects of different doses of nasally administered antigens are warranted. An alternative possibility for treating ongoing autoimmune disease could be nasal administration of antigens with high tolerogenic efficacy, like altered peptide ligand [41].

The transient activation of CD4⁺ T-cells that precedes the development of tolerance *in vivo* in animals receiving autoantigens nasally also raises problems. Ongoing disease may be exacerbated upon nasal antigen administration in the acute phase of disease. For the application of nasal tolerance in clinical medicine, it is important to identify measures that counteract, but do not activate, an adverse immune response.

Conclusions

Nasal administration of soluble antigens or peptides with defined T-cell epitopes is receiving much attention as a means of specifically down-regulating pathogenic T-cell reactivities in autoimmune disorders. As discussed before,

the nasal approach is very efficient in terms of inducing T-cell hypo-responsiveness and clinically relevant disease suppression. Given the very small doses required using this approach which are still very effective, the nasal route should be considered as another possible approach for developing antigen-specific therapy of autoimmune diseases in human. Depending on the quality and quantity of the antigens used, the mechanisms of nasal tolerance are either anergy/deletion of antigen-specific T-cells or induction of IL-4 and/or TGF-β related regulatory mechanisms, depending on the property and dosage of antigens administered.

Given the probability that multiple autoantigens or epitopes are involved in the pathogenesis of autoimmune diseases, and the differential tolerogenic effects of antigens, antigen-specific therapy should be designed on the basis of activating regulatory elements which can non-specifically suppress inflammatory responses in the peripheral lymphoid system and/or in the target organ. Only those antigens or peptides with high efficacy in inducing nasal tolerance by inducing regulatory elements, without further activation of autoreactive T-cells, are candidates to be useful in clinical medicine.

Future directions

Nasal tolerance induction using autoantigens or defined T-cell epitopes is still at an early stage of development. Ideal antigens or peptides which will have a chance to be used for induction of nasal tolerance in clinical medicine should have the following properties: (1) high efficacy in inducing nasal tolerance, thereby reducing the dose required for nasal tolerance induction and reducing the risk of non-specific damage to the nasal and bronchial tract mucosa; (2) induction of regulatory elements. Given the complexities of autoantigens involved in the pathogenesis of autoimmune diseases and the differential tolerogenic effects of autoantigens and peptides, antigen-specific therapy should be designed, based on activating regulatory elements, which can non-specifically suppress inflammatory responses in the peripheral lymphoid system and/or in the target organ; (3) no activation of autoreactive T-cells. Nasal tolerance induction may involve an active process, i.e., activation of T-cells may precede tolerance induction. When used to treat ongoing disease, it is critically important that the induction of nasal tolerance does not transiently exacerbate ongoing disease. None of the antigen-specific therapies hitherto tested meet with all these standards. It is thus very clear that antigen-specific therapy of autoimmune diseases is still at an early stage of its development. However, we anticipate that in the future, such 'smart bombs' can be useful in clinical medicine.

An alternative approach to antigen-specific treatment in

the future could be the combined administration with cytokine(s) by the nasal route. Since co-stimulation of CD4⁺ T-cells with antigen and IL-4 plays a dominant role in the induction of IL-4-producing TH₂-cells *in vitro* and *in vivo* [69], IL-4-induced immune deviation might be a therapeutic regimen capable of preferentially modulating the responses of autoantigen-specific T-cells *in vivo*, even in the absence of a clear definition of the structures of the autoantigens and the fine specificity of the T-cells involved in pathogenic immune reactions. The combination of antigen-specific therapy with IL-10 could be even more attractive, given the strong down-regulatory actions of IL-10 on antigen-specific TH₁ responses and its limited toxicity.

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