Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression

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Abstract

Mucosal administration of antigens in experimental animals leads to the induction of peripheral T cell tolerance. We have previously reported that in H-2b mice, intranasal (i.n.) or oral administration of a peptide containing the immunodominant T cell epitope will down-regulate the function of CD4+ T cells reactive with Der p 1, a major target antigen in both B and T cell responses to house dust mite. In the present study we have investigated the tolerogenicity of peptides containing both dominant and subdominant determinants when given i.n. to naive mice. Induction of tolerance by the nasally administered immunodominant peptide leads to a diminution in all T cell-derived cytokines and modulation of delayed-type hypersensitivity responses, but IgE production did not seem to be affected, furthermore the induction of T cell tolerance was stable, lasting beyond 6 months. We have also examined the specificity of intramolecular epitope suppression which is a feature of mucosal tolerance induced by nasally administered peptides and demonstrate that regulatory CD4+ T cells may exert their suppressive effect by linked recognition of epitopes on the same or neighbouring antigen-presenting cells.

Introduction

Soluble protein antigens encountered through the respiratory or gastrointestinal tracts do not elicit strong systemic immune responses but induce a state of antigen-specific unresponsiveness which is commonly referred to as mucosal tolerance. It is characterized by a diminution of T cell effector responses and consequently antibody synthesis. As regards the cellular mechanisms responsible for the development of tolerance, it appears that feeding high doses of antigen can lead to the deletion of antigen-specific T cells (1), anergy (2–4) or a shift in the cytokine production of T cells (5). Similarly, tolerance to inhaled antigens can arise as a result of anergy or immune deviation (6,7).

Early studies on mucosal tolerance were restricted to analysis of cellular responses to whole protein antigens, but it now appears that immunogenic peptides which contain T cell epitopes can also act as potent tolerogens in vivo (8–12). Peptides derived from self antigens delivered in tolerogenic form through mucosal surfaces can prevent the induction of, or reduce the severity of, clinical symptoms associated with various autoimmune diseases (12–14). Furthermore, we have previously reported that the intranasal (i.n.) or oral administration of a single immunodominant peptide derived from the house dust mite (HDM) protein Der p 1, when given prior to immunization with the whole protein, can induce peripheral tolerance in both naive (15,16) or sensitized mice (15). The non-responsiveness is characterized by a reduction in IL-2 production by both lymph node and splenic T cells, and their failure to provide cognate help for antibody production (15).

A feature of peptide-induced mucosal tolerance is that treatment with a single immunogenic peptide can abrogate T cell responses to all the epitopes on an antigen...
have investigated further the specificity and duration of the antigen-specific immune response. In the present study we only poorly in the presence of IL-4 (20). Test supernatants
recognize of tolerogenic antigen which helps to block an The CTLL-2 cell line proliferates maximally with IL-2 but
bystander suppression to an unrelated antigen, peptide-

µ
µ

IFN-

γ

and TGF-β. All cytokines were measured in culture supernatants taken at 24 h intervals over a 96 h period. IL-2 and IL-3 were usually measured at 24 h, IL-5, IFN-γ and TGF-β at 48 h, and IL-4 at 96 h.

The CTLL-2 cell line proliferates maximally with IL-2 but only poorly in the presence of IL-4 (20). Test supernatants (50 μl volumes) were added to 5×10^3 CTL-2 cells (50 μl) per well and cultured for 24 h at 37°C and pulsed for 6 h with [3H]thymidine (1 μCi; Amersham, Amersham, UK).

FDC-P1 cells proliferate maximally to IL-3 and granulocyte macrophage colony stimulating factor but poorly to IFN-γ. Cells (2×10^5) were cultured with test supernatants for 24 h for FDC-P1 cells and 96 h for CT.4S cells and pulsed with [3H]thymidine.

CT.4S cells proliferate in response to IL-4, but only poorly to IL-2 (20). Cells (2×10^5) were cultured with test supernatants for 24 h for FDC-P1 cells and 96 h for CT.4S cells and pulsed with [3H]thymidine.

IFN-γ was measured using an ELISA assay. Briefly, rat anti-mouse IFN-γ mAb (R4-6A2; PharMingen, San Diego, CA) was coated onto Immulon-2 plates overnight and washed with PBS containing 0.05% Tween 20 (Sigma). Plates were blocked with 10% BSA dissolved in PBS. After washing, culture supernatants were added to the plates and incubated for 2 h at room temperature. Plates were washed and the presence of bound IFN-γ detected by biotinylated anti-mouse IFN-γ (XMG1.2; PharMingen). Plates were developed using streptavidin–alkaline phosphatase (Sigma) with 3,3′-5,5′-tetramethyl benzidine (Sigma) as the substrate and plates were read at 405 nm. The concentration of IFN-γ was measured from a standard curve using recombinant IFN-γ (PharMingen).

IL-5 was measured in 48 h culture supernatants by ELISA using a commercially available kit (Endogen IL-5) and was performed following the manufacturer’s directions. Standard recombinant IL-5 preparations were included in each assays.

TGF-β1
The growth of the mink lung epithelial cell line MV-1Lu is arrested in the presence of TGF-β1. Test supernatants (50 μl volumes) were added to 2×10^5 MV-1Lu cells (50 μl) per well and cultured for 24 h at 37°C and pulsed for 6 h with [3H]thymidine. The concentration of TGF-β1 in supernatants was determined from a standard curve using purified TGF-β1.

Skin testing for delayed-type hypersensitivity (DTH) responses
Mice received either PBS or p111–139 nasally on three consecutive days and 9 days later mice were immunized with 100 μg Der p 1/CFA. After 7 days mice were injected intradermally with 10 μg Der p 1 in 10 μl PBS. Ear swelling was measured after 24 h and the increments were obtained by subtracting values of the test groups from those control mice who received either PBS or p111–139 alone.

Passive cutaneous anaphylaxis (PCA) assay
Mice were treated nasally with PBS or a tolerogenic dose of peptide 111–139 and then immunized 9 days later with Der
p 1/CFA. After 21 days mice were bled and the serum was serially diluted in doubling dilutions in PBS. Serum was injected intradermally into the back of an adult male Sprague-Dawley rat. After 24 h the rat was anaesthetized and injected with 10 µg of Der p 1 in saline containing Evans blue. Serum from Der p 1 hyperimmunized mice was used as a positive control. A positive result was scored at the serum dilution which gave a wheal of 10 mm or larger.

**Statistical analysis**

The Student's t-test and the Mann–Whitney U-test was used to analyse the statistical significance of the experimental data where appropriate.

**Results**

**Comparison of peripheral non-responsiveness in H-2b mice induced by i.n. administration of peptides containing immuno-dominant or minor T cell epitopes of Der p 1**

We have previously described the dominant or subdominant T cell epitopes of the HDM allergen Der p 1 which are recognized following immunization of H-2b mice with affinity-purified protein emulsified in CFA (16). Peak responses are observed to peptide 110–131, while weaker responses are observed to 81–102, 197–212 and 21–49. The ability of Der p 1-derived peptides containing either the immunodominant (p111–139) or a minor (81–102) epitope to inhibit antigen-specific CD4⁺ T cell responses when administered nasally was investigated. In all the experiments performed in this report, control mice received PBS i.n., but the specificity of tolerance induction of this model has been previously reported (15). Mice were treated with either PBS or with 100 µg of p111–139 or p81–102 on three consecutive days and immunized with Der p 1/CFA 14 days after the last peptide treatment. The capacity of LN T cells to secrete various cytokines following in vitro stimulation with the Der p 1 protein 10 days after the parenteral challenge was examined. All cytokines were measured at various time points from 24 to 96 h in order to determine the optimal secretion and for simplicity cytokines shown in Fig. 1 are only from the peak response. T cells from control mice were still highly responsive to the Der p 1 protein in vitro and could secrete IL-2, IL-3, IL-5, IFN-γ but with little or no IL-4, while T cells from mice treated with HDM peptide displayed down-regulated cytokine responses in vitro for all the cytokines measured (Fig. 1). In addition, we did not observe any increase in TGF-β1 secretion following tolerance induction to nasally administered peptides (Fig. 1). The level of tolerance achieved with the minor epitope was not as complete as that obtained with the dominant peptide, with the minor epitope only reducing responses to 50–60% of that observed in the control animals (Fig. 1). In all of the remaining experiments we have focused on the secretion of IL-2 by LN T cells since this is the cytokine that is most markedly inhibited in T cells following mucosal tolerance and is a general feature of peripheral T cell tolerance.

**Peptide tolerance affects DTH responses but not IgE responses**

The above results indicate that T cell responses are markedly reduced following induction of tolerance to nasally adminis-
Fig. 1. Modulation in the secretion of T cell-derived cytokines following tolerance induction to nasally administered peptides. Mice were treated i.n. with either PBS (●), p111–139 (▲) or p81–102 (□) and 14 days later challenged with Der p 1/CFA. LN cells from five mice per group were cultured in vitro with Der p 1 and cytokines measured after 24 or 48 h. Data shows the mean response ± SD for each of the cytokines. Data have been compiled from at least three experiments.

However, mice that were pretreated with p111–139 showed down-regulated responses to the protein and to p197–212 (Fig. 5A and B). The remaining mice were immunized with 50 µg of 197–212/incomplete Freund’s adjuvant (IFA) 3 weeks after the Der p 1 challenge, and after a further 10 days LN cells from both groups were tested for responses to the dominant and minor epitopes. When analysed, T cells from control mice and peptide-tolerated mice displayed equivalent responses to the minor epitope 197–212 (Fig. 5C and D), while T cell responses in both groups to the dominant epitope were still down-regulated.

**Bystander suppression**

The previous experiments demonstrate that the nasally administered immunodominant peptide can induce regulatory T cells that can block T cell responses to epitopes linked on the
Fig. 2. Tolerance affects T but not B cell responses to Der p 1. (A) Mice were treated with PBS (n = 5) or a tolerogenic dose of p111–139 (n = 6) i.n. and 9 days later mice were immunized with Der p 1/CFA. One week later mice were injected intradermally in the ear with 10 µg Der p 1 in saline. Twenty four hours later skin swelling was measured with a micrometer on the ear injected with antigen and that uninjected. The two values were subtracted from each other to give increments of ear swelling. Control animals received either PBS or p111–139 i.n. only in order to establish background swelling responses. (B) Sera from control or peptide tolerized mice were measured for the presence of allergen-specific IgE by PCA. Serial dilutions of serum were injected intradermally into the back of a Sprague-Dawley male rat and 24 h later the rat was injected with a solution containing 10 µg Der p 1 in Evans Blue. Wheal responses were measured and scored positive responses if they were >10 mm. Data shows the mean antibody dilution to give a positive response.

Fig. 3. Peripheral tolerance induced by i.n. peptide persists for long periods in vivo. Mice were treated with either PBS (●) or p111–139 and immunized after 2 weeks (△) or received p111–139 i.n. and rested for 6 months before being immunized with Der p 1/CFA (□). Ten days after the immunization LN cells from each group were cultured in vitro with the p111–139. Data shows the mean IL-2 response from five mice per group ± SD.

same protein. We wanted to examine if these regulatory T cells may also block immune responses to unrelated antigens in vivo. Mice were tolerated with the dominant peptide as usual but on day 14 post-treatment mice were co-immunized with Der p 1 and OVA in CFA. Ten days later LN cells were cultured in vitro and responses tested to Der p 1 or OVA separately. As shown in Fig. 6, T cell responses to Der p 1 were clearly abrogated (P < 0.05) but although some decrease in T cell responses was observed to the bystander antigen OVA, this was not statistically significant (P > 0.1).

Discussion
The delivery of antigens through mucosal surfaces is an efficient way of inducing antigen-specific T cell non-responsiveness, which is referred to as mucosal tolerance. We have previously reported that i.n. administration of the immunodominant peptide (residues 111–139) derived from the Der p 1 allergen of HDM can specifically inhibit CD4+ T cell responses to the whole antigen when administered to naive or sensitized H-2b mice (15). Several mechanisms have been identified to explain the loss of T cell antigen-reactivity following the induction of mucosal tolerance. Clonal deletion of antigen-reactive T cells has been observed following high-dose feeding of protein antigens to TCR transgenic mice (1), while others have suggested a role for clonal anergy of CD4+ T cells (2,3). In addition there is evidence for active suppression in oral tolerance mediated by TGF-β1 CD8+ T suppressor (Tₜ) and, more recently, immunoregulatory CD4+ T cells that secrete Tₜ2-type cytokines (e.g. IL-4, IL-10 and TGF-β1) have been isolated from the Peyer’s patch and mesenteric lymph node following feeding with low doses of myelin basic protein (MBP) (5). However, the role for CD8+ Tₜ cells in oral tolerance is controversial, since several groups have failed to observe a role for such cells in the induction or maintenance of oral tolerance (2,3,21).

Studies on the immune response to nebulized antigens have revealed that allergic sensitization is naturally avoided through a shift in cytokine production (or immune deviation) by specific CD4+ T cells during the course of a primary immune response to the antigen (7). There is evidence that regulatory CD8+ Tₜ cells become activated following inhalation of protein antigen in experimental animals which can block antigen-specific IgE synthesis in vivo (22–25). However, it was recently shown that CD8+ T cells activated by inhaled antigen may play a role in mediating airway hypersensitivity (26). In addition immune deviation was shown to be responsible for the protection of non-obese diabetic mice from autoimmune diabetes following nasal administration of a cocktail of GAD65 peptides (27).

Induction of tolerance to high-dose nasally administered peptide is preceded by a transient response by CD4+ T cells
Mucosal T cell tolerance

Fig. 4. Molecular requirements for intramolecular epitope suppression. Mice were treated with either PBS (A–C) or p111–139 (D–F), i.n. and 2 weeks later mice were challenged with either Der p 1/CFA (A and D), p111–139/CFA (B and E) or p81–102/CFA (C and F). Data shows the 24 h IL-2 response from LN cultures of individual mice.

Fig. 5. T cells specific for minor epitopes are not deleted or anergized following i.n. treatment with the immunodominant peptide. Mice were treated i.n. with either PBS (●) or p111–139 (□) and 2 weeks later immunized with Der p 1/CFA. After 10 days half the mice of each group were sacrificed and LN cells tested for responses to (A) Der p 1 and (B) p197–212. The remaining mice were then immunized with p197–212/IFA 3 weeks later and after a further 7 days LN cells were cultured in vitro with p197–212 (C and D). Data shows the IL-2 response from individual mice.

We have not identified a role for CD8+ T cells in this contrast to studies on oral tolerance to MBP where TGF-β secretion seems to be an important mechanism for regulating responses of self-reactive CD4+ T cells. In the model studied here, the production of all T-cell-derived cytokines was down-
regulated following tolerance induction with a peptide containing either the dominant or a minor T cell epitope. Although the level of T cell inhibition achieved with the minor T cell epitope was not as profound as that obtained with the immunodominant epitope, our results are in agreement with other reports (9,13) that demonstrate a hierarchy of potency of peptides as tolerogens which reflects their immunogenicity in vivo. The finding that there is a loss of cytokine production by antigen-specific T cells following tolerance induction is consistent with a previous study on peptide-induced peripheral tolerance (28). However, there may be distinct mechanisms which operate in the induction of mucosal tolerance through the respiratory tract and this may be dependent upon the dose, the frequency and the nature of the antigen administered, as has been shown for oral tolerance (29).

Although the nasal administration of soluble peptide appears to induce a strong primary immune response (6), the functional outcome of this response is qualitatively different had the same peptide been administered in conjunction with adjuvant. We have previously suggested that mucosal tolerance to high-dose peptide induces a population of regulatory T cells which display an anergic phenotype in vitro (6). The cells remain in the peripheral circulation but can no longer act as classical Th cells (15,16) but instead adopt an immunoregulatory role and function to modulate rather than promote antigen-specific immune responses. Furthermore, these regulatory T cells appear to be maintained in the peripheral circulation for long periods, as evidenced by their capacity to down-regulate T cell-dependent immune responses, such as cytokine secretion and DTH responses to Der p 1. Moreover, the regulatory T cells appear to be functional even 6 months after the original peptide treatment. This finding is in agreement with the stability of oral tolerance in mice where a single feed of protein to a naive animal can lead to life long antigen-specific T cell non-responsiveness (30).

Although antigen-specific T cell responses were modulated following tolerance induction, the levels of specific IgE antibody were not affected. The limitation to this study was that most immunizations were made in CFA which should bias for a Th1 dominant immune response. Nevertheless, we have also observed similar findings when mice were also immunized under Th2-type conditions. Therefore, the mucosally delivered peptide tolerogen appears to affect the function of CD4+ Th cells while B cell function in the short term does not appear to be modulated. These findings are consistent with studies which have examined immunological functions of patients who have undergone conventional allergen immunotherapy (31), where T cell functions were demonstrated to have been modulated without affecting the levels of specific serum IgE in the short term.

A feature of mucosal tolerance induced by nasally administered peptide is that a single peptide can inhibit responses to all epitopes on an antigen (13–17). In the model system studied here, the induction of tolerance to the immunodominant epitope of Der p 1 can down-regulate T cell responses to all four epitopes on the antigen when mice are immunized with the whole protein in adjuvant (15). However, it was possible to uncouple tolerance to the minor epitopes by immunizing tolerant mice with a peptide containing only the subdominant determinant, which is consistent with previous studies on peripheral T cell tolerance (8). However, it was possible to rescue a T cell response to a minor epitope of Der p 1 in mice that had been rendered tolerant to the dominant epitope and who had been previously immunized with the whole protein (Fig. 5). If such mice were immunized with the subdominant peptide in adjuvant, 3 weeks after the initial protein immunization, then the previously tolerant mice displayed equivalent responses to the subdominant epitope as controls.

Taken together, these observations suggest that the T cells specific for the minor epitopes are not deleted or anergized after nasal administration of the immunodominant peptide, but should be competent to respond to their epitopes following immunization with the protein. Yet this is not the case. Thus the intramolecular epitope suppression observed in our model is probably due to local suppression mediated by regulatory CD4+ T cells through the recognition of linked epitopes on the same antigen. However, rather than killing the T cells specific for minor epitopes, we suggest that the regulatory T cells may merely block their growth. Once the immune response to the protein has waned, the regulatory T cells would return to a resting state since their epitopes would not be presented on APC. Thus when tolerant mice are
reimmunized with a peptide containing only the subdominant determinant, T cells specific for the epitope would be allowed to expand normally since they would no longer be under the control of the regulatory CD4\(^+\) T cells. The phenomenon of linked suppression is not new and has been observed in a murine model of mAb-induced transplantation tolerance (18,19). Regulatory CD4\(^+\) T cells induced by this tolerance protocol can mediate local suppression of allo-reactive T cells by linked recognition of antigen possibly on the same APC and can generate some bystander suppression to a third party antigen (18). Although we observed some inhibition of T cell responses to a bystander antigen in our model of peptide induced mucosal tolerance, the level of inhibition was not statistically significant.

We propose the following model to explain how intramolecular epitope suppression might be induced in mucosal tolerance. Following immunization of tolerant mice with the intact protein, APC in the draining LN will present peptides specific for the regulatory T cells and for those T cells recognizing the subdominant epitopes. The APC may therefore act as a bridge to bring the regulatory cells into close vicinity with the naive T cells of differing specificity. In such an environment the regulatory T cells may exert their inhibitory effects to other T cells either through the release of inhibitory cytokines or by negative signalling via direct membrane interactions. Alternatively, the regulatory T cells may compete for the local production of IL-2 by ‘mopping up’ excess IL-2 and thus prevent expansion of the naive T cells specific for the subdominant epitopes, as has been suggested by a human model of T cell anergy (32). The induction of bystander suppression in this model was not convincing, but this may be due to a problem in local antigen presentation, in that different APC may present the epitopes for the two antigens and these APC may be separated by a sufficient distance in vivo that enables the OVA-reactive T cells to escape the regulatory actions of the p111–139-specific T cells in vivo.

The observation reported here that T cell tolerance to i.n. peptide is long lasting may facilitate this route of antigen delivery in immunotherapy (33). Since a single immunogenic peptide alone can establish T cell tolerance by inducing a population of regulatory T cells, it may not be necessary to vaccinate with multiple T cell epitopes. In addition, further studies may help identify the molecular basis for tolerance induction and may also provide an insight into the functional properties of mucosal APC that make them effective in tolerance induction.

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
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<td>Der p 1</td>
<td>Dermatophagoides pteronyssinus 1</td>
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<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
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<tr>
<td>HDM</td>
<td>house dust mite</td>
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<tr>
<td>IFA</td>
<td>incomplete Freund’s adjuvant</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PCA</td>
<td>passive cutaneous anaphylaxis</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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