

Kinetics and mode of peptide delivery via the respiratory mucosa determine the outcome of activation versus T_H2 immunity in allergic inflammation of the airways

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Background: Specific immunotherapy involving systemic injection of allergen, though highly effective, can cause severe side effects due to IgE-mediated activation of effector cells. Allergen-derived peptides might provide a safer alternative. We have investigated the use of mucosally delivered peptide to induce CD4⁺ T_H2 cell tolerance and thus protect against allergen-induced airway inflammation.

Objective: The purpose of this study was to investigate whether intranasal administration of an allergen-derived peptide, either alone or adsorbed to chitosan, can prevent the induction of T_H2-mediated pulmonary inflammation after sensitization and challenge of the airways with allergen.

Methods: Mice were given (intranasally) a peptide containing an immunodominant epitope of the *Dermatophagoides pteronyssinus* (Der p) 1 allergen, either as soluble antigen or adsorbed to chitosan, before sensitization and allergen challenge. Pulmonary inflammation, antigen-specific CD4⁺ T-cell responses, and antibody levels in sera were then determined.

Results: Mice given peptide adsorbed to chitosan had significant reductions in airway eosinophilia, which correlated with reduced levels of IL-4 and IL-5 in the bronchoalveolar lavage fluid. There was decreased recruitment of activated CD4⁺ T cells into the airways after allergen challenge, which correlated with a loss of Der p 1-specific T-cell cytokine responses in the periphery and the localized production of IL-10 by antigen-specific T cells in bronchial lymph nodes. Induction of peripheral T-cell tolerance was preceded by transient T-cell activation and IFN- γ production.

Conclusion: Our data demonstrate that suppression of airway inflammation by intranasal administration of peptide antigen adsorbed to chitosan is initiated by transient T-cell activation and maintained by the production of IL-10 by antigen-specific T cells in the draining lymph nodes. (*J Allergy Clin Immunol* 2002;110:883-90.)

Key words: Allergy, tolerance, immunotherapy, in vivo animal models

Allergic asthma is characterized by elevated serum IgE levels, airway hyperresponsiveness, and bronchial inflammation after exposure of the airways to inhaled allergens. CD4⁺ T_H2 cells expressing an activated memory phenotype and the cytokines IL-4, IL-5, IL-9, and IL-13 have been isolated from the bronchoalveolar lavage fluid (BALF) of acute allergic asthmatic individuals and are associated with the disease pathology.^{1,2}

The type of effector T-cell response generated in vivo is determined by antigen concentration, route of delivery, and the presence of inflammatory signals, which influence antigen-presenting cell (APC) activation, upregulation of costimulatory molecules, and cytokine production. Inflammatory signals are required for maturation of dendritic cells (DCs) in the airway mucosa, upregulation of MHC class II proteins, the costimulatory molecules (CD80 and CD86), and chemokine receptor CCR7, which is critical for migration of DCs to the draining lymph nodes.^{3,4} The interaction between APCs and CD4⁺ T cells during antigen presentation and the cytokine milieu influence the primary response generated.⁵ CD4⁺ T-cell activation involves ligation of the specific T-cell receptor (TCR) by MHC class II peptide complexes. In the absence of APC-derived costimulatory signals, TCR ligation leads to functional inactivation and anergy.⁶ As in activation, a critical threshold level of signaling needs to be reached for induction of T-cell tolerance, which is preceded by and critically dependent on an initial state of transient activation, upregulation of TCR and CD69 expression, and increased proliferative and cytokine responses, after antigen-specific TCR ligation.⁷ Peripheral T-cell tolerance can be induced by the mucosal delivery of soluble antigen.⁸ Studies in animal models have demonstrated that intranasal administration of allergens to the upper respiratory tract before parenteral immunization can suppress the induction of peripheral allergen-specific lymphoproliferative T-cell responses and IgE production.⁹⁻¹¹ The effect on T cell-mediated inflammatory responses of the lower airways requires further investigation. Clinical studies have demonstrated that subcutaneous immunotherapy using peptides can induce peripheral T-cell tolerance with attenuation of allergen-induced late-phase cutaneous, but

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Abbreviations used

APC: Antigen-presenting cell
BALF: Bronchoalveolar lavage fluid
DC: Dendritic cell
Der p 1: Group 1 allergen derived from *Dermatophagoides pteronyssinus* species
pDC: Pulmonary dendritic cell
TCR: T-cell receptor

not asthmatic, reactions.¹² In this study, we investigated whether intranasal administration of a peptide encoding an immunodominant T-cell epitope of Der p 1,¹³ a major aeroallergen of house dust mite, can suppress induction of CD4⁺ T-cell responses and airway eosinophilia after allergen challenge in a murine model of pulmonary inflammation.

Peptide absorption across the nasal mucosa occurs principally by passive diffusion across intercellular barriers formed by tight junctions. To reach a critical TCR activation threshold for tolerance induction, we administered Der p 1; p111-139, the immunodominant T-cell epitope of Der p 1, as a soluble peptide adsorbed to chitosan. Chitosan is a nontoxic, biocompatible, and biodegradable mucopolysaccharide that enhances the level of absorption of compounds delivered via the nasal mucosa.^{14,15} This is due in part to the bioadhesive properties of chitosan but is also due to the transient opening of the tight junctions between epithelial cells, which form a barrier to the external environment. Because this increase in absorption occurs in the absence of tissue damage or inflammation, the delivery of peptide adsorbed to chitosan could facilitate tolerance induction by increasing the level of peptide released systemically.¹⁶

METHODS**Animals**

C57BL/6 mice, 6 to 8 weeks of age, were used. Animals were purchased from Harlan. Animal care and experimental procedures were conducted in accord with the animal ethics regulations of the Home Office in the United Kingdom.

Antigens

The house dust mite allergen Der p 1 was affinity-purified from spent mite medium through use of the monoclonal antibody 4C1 (Indoor Biotechnologies Ltd). The synthetic peptide Der p 1 p111-139 (FGISNYCQIYPPNANKIRELAQPQRYCR) was synthesized with a free carboxyl terminus and HPLC-purified (Advanced Biotechnology Centre, Imperial College School of Medicine, Charing Cross Hospital, London). The chitosan–Der p 1 p111-139 peptide solution was made up as follows: 2% chitosan in acetic acid (0.5% w/v; Vanson Inc) was dissolved by sonication, diluted further in d H₂O (0.4% w/v), and filter-sterilized. The chitosan was used at a final concentration of 0.25% w/v adsorbed to a minimum of 5 mg/mL peptide.

Immunization and tolerance induction

The following optimized treatment regimes were used: Mice were lightly anesthetized under ether, and groups of mice were treated by the intranasal administration, on each of 5 consecutive

days, of a 20- μ L volume of endotoxin-free saline solution containing 100 μ g of either Der p 1 p111-139 peptide or chitosan adsorbed to Der p 1 p111-139 peptide. The control groups received either saline solution or chitosan alone. Mice were immunized 14 and 16 days after the last intranasal treatment by the intraperitoneal injection of Der p 1 (10 μ g) in saline solution adsorbed to an equal volume of aluminum hydroxide adjuvant (Imject Alum; Pierce). On days 41 and 43, mice were challenged by intratracheal instillation of Der p 1 (20 μ g) into the airways in 50 μ L saline solution and killed after 24 hours by means of a lethal dose of Avertin. To determine antibody levels in sera, mice were bled from the tail vein before being killed. Blood samples were centrifuged at 2000g; sera were collected and stored at -70°C . For the mechanistic studies, mice were treated intranasally for 3 consecutive days with saline solution, peptide, or peptide adsorbed to chitosan; after either 48 or 96 hours, the mice were killed and their cervical lymph nodes were removed.

T-cell proliferative responses

We prepared single-cell suspensions from the spleen or lymph nodes by teasing the tissue apart and passing it through a fine wire mesh to remove cell debris after red blood cell lysis. Whole cell populations were cultured in RPMI 1640 medium (Gibco) supplemented with 5% FCS, 50 μ mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, and 10 U penicillin/streptomycin (Gibco). To measure specific T-cell proliferative responses, cells (1×10^5 /well) were cultured in round-bottomed, 96-well microtiter plates (Costar, Corning Incorporated) in medium with Der p 1 p111-139 peptide (5, 20 μ g/mL) or Der p 1 (10 μ g/mL). To control for T-cell viability, cells were polyclonally stimulated by being cultured in wells containing immobilized anti-CD3 (145-2C11) and anti-CD28 (37.51; 5 μ g/mL). After 72 hours, cells were pulsed with 3H-thymidine (1 μ Ci/well) and the levels were incorporated after a further 16 hours of culture, as determined by liquid scintillation counting.

Cytokine production

Single-cell suspensions prepared from spleen or lymph nodes were cultured (5×10^6 /mL) in complete media containing either Der p 1 or Der p 1 p111-139 peptide (10 μ g/mL). Control wells contained (a) medium alone or (b) an irrelevant antigen;OVA (10 μ g/mL), or wells were coated with anti-CD3 and anti-CD28 (5 μ g/mL) antibodies. After 72 hours supernatants were collected; they were stored at -70°C and later assayed in duplicate for the presence of the cytokines IL-4, IL-5, IL-10, IL-13, and IFN- γ through use of ELISA. Antibodies were purchased and used according to the instructions of the manufacturer (IL-4, IL-10, and IL-13, R & D Systems; IL-5 and IFN- γ , Pharmingen).

Measurement of eosinophil and cytokine levels in BALF

Immediately after sacrifice, the trachea were exposed and cannulated with 27-gauge silicon tubing attached to a needle and syringe. The lungs were instilled through the trachea with 500 μ L cold PBS, and the BALF was withdrawn. The volume of BALF collected was measured for each sample. To determine BALF cell numbers and composition, samples were centrifuged (700g, 5 minutes, 4°C) and cells were washed (PBS/10% FCS), counted, and resuspended at 1×10^6 /mL. BALF (1×10^5) cells in 100 μ L were cytopun onto poly(L-lysine)-coated slides (3 minutes at 300 rpm by means of a cytocentrifuge). The slides were air-dried, fixed in methanol, and stained through use of Diff-Quik (Dade-Behring). To determine cytokine levels in BALF, samples were stored at -70°C . Cytokines were analyzed through use of ELISA.

Determination of Der p 1–specific antibody levels in sera

Antibody levels in sera were determined through use of ELISA. Unless otherwise indicated, serum samples and antibodies were diluted in PBS/1% BSA and wells were washed between incubation steps with PBS/0.05% Tween-20. Round-bottomed, 96-well microtiter plates (Costar) were coated overnight at 4°C either with rat antimouse IgE (R35-72; Pharmingen) for detection of specific IgE levels or with Der p 1 (5 µg/mL) diluted in carbonate buffer (0.1 mmol/L NaHCO₃, pH 8.2) for detection of specific IgG1 and IgG2a. After washing, nonspecific binding was blocked by incubating with PBS/3% BSA for 2 hours at 37°C. Plates were washed, and serial dilutions of serum samples were added in duplicates (IgE, 1:5, 1:10, and 1:20; IgG1, 1:50, 1:100, and 1:500; IgG2a, 1:10, 1:20, and 1:50). The plates were incubated overnight at 4°C. After washing, biotinylated rat antimouse IgG1 (LO-MG1-2; Serotec) or rat antimouse IgG2a (Lo-MG2a-7; Serotec) was added at 1000 ng/mL; plates were then incubated for 2 hours at room temperature. To determine Der p 1–specific IgE levels in sera, Der p 1 (10 µg/mL) was added and plates were incubated at 37°C for 60 minutes. After washing, plates were incubated for an additional 45 minutes with biotinylated anti-Der p 1 mAb 4C1 (1:1000 dilution). Bound biotinylated antibodies were detected by being incubated with streptavidin-peroxidase for 45 minutes at room temperature and visualized through use of the TMB substrate. Plates were read in a microtiter reader at 450 nm through use of the MRX microplate reader (Dynatech) and Revelation software.

Statistical analysis

The data represent mean values ± SEMs. There were 5 mice in each group. Differences between mean values were analyzed by 1-way ANOVA through use of either the Dunnett multiple comparison parametric test or the 2-tailed Student *t* test (antibody levels) with InStat (GraphPad Software). *P* values less than or equal to .05 were considered statistically significant.

RESULTS

Cytokine production by allergen-reactive T cells isolated from mice treated prophylactically by intranasal administration of peptide before sensitization and challenge with allergen

We investigated whether intranasal administration of peptide leads to the induction of tolerance and a loss of cytokine production or a T_H1/T_H0 shift in the phenotype of the effector T-cell populations. For this purpose, cells isolated from the spleens (Fig 1, A) or lymph nodes draining the airways (Fig 1, B) of Der p 1–sensitized and –challenged mice were restimulated *in vitro* with Der p 1; levels of IL-5, IL-13, IL-10, and IFN-γ in culture supernatants were then determined through use of ELISA. Investigation of systemic responses revealed reductions in both T_H2 (IL-5 [65%] and IL-13 [62%]) and T_H1 (IFN-γ [75%]) cytokines in peptide-chitosan–treated mice compared with control mice, suggesting that Der p1–reactive T cells in the spleen were tolerized (Fig 1, A). Systemic cytokine responses were not suppressed in mice treated with peptide alone. Furthermore, these mice exhibited marked increases in levels of the T_H2 cytokines IL-5 (3-fold) and IL-13 (7-fold)

on stimulation of cells obtained from draining lymph nodes with Der p 1. The differential effect of peptide and peptide adsorbed to chitosan on recall responses in lymph node cultures suggest that the kinetics of mucosal delivery might influence the extent to which naive T cells are primed at sites of antigen exposure.

There is increasing evidence that tolerance induced by mucosal administration of soluble antigen is maintained by antigen-specific T regulatory cells through the production of immunosuppressive cytokines. Increases in IL-10 production in lymph node cultures were observed on restimulation with Der p 1 in mice treated with peptide (70%) or peptide adsorbed to chitosan (51%) compared with control mice. IL-10 production was antigen-dependent. Nevertheless, reductions in T_H2-mediated eosinophilia on rechallenge with antigen were associated with increases in the ratio of IL-10 to IL-5 produced (Fig 1, C) rather than with increases in IL-10 alone or increases in the ratio of IFN-γ to IL-5 (data not shown), suggesting that in the context of a T_H2-driven response, mechanisms influencing the initial state of T-cell tolerance induction might play a role in maintaining a state of antigen-specific nonresponsiveness. IL-10 levels produced by cells isolated from the spleen did not differ markedly between groups, suggesting that IL-10 might play a greater role in maintaining tolerance at sites of T-cell priming.

Effect of intranasal peptide treatment on antigen-specific immunoglobulin levels induced after systemic immunization

Because the induction of IgE and IgG1 synthesis by B cells requires a cognate interaction with antigen-specific CD4⁺ T_H2 cells, we investigated whether the tolerization of T cells reactive with an immunodominant region of Der p 1 by intranasal administration of Der p 1; p111-139 peptide was sufficient to inhibit antibody production after immunization with the intact Der p 1 allergen. Mice given peptide intranasally showed minor reductions in Der p 1–reactive IgG1 and IgG2a levels in sera compared with control mice given PBS. IgE levels, however, were not reduced (Fig 2). Mice given peptide-chitosan showed no reductions in serum IgG levels, though IgE production was increased (*P* < .05). Comparable increases in IgE production were observed in mice given chitosan alone. These increases might reflect the inhibitory effect of chitosan on IFN-γ production (Fig 1, A and B) and consequently an upregulation of IL-4–dependent humoral responses. This suggests that chitosan might not be the most appropriate vehicle for allergen therapy.

Effect of intranasal peptide administration on eosinophil levels in Der p 1 allergen-challenged airways

The infiltration of eosinophils into the airways after allergen challenge is a major pathologic feature of allergic asthma. To determine whether the intranasal delivery

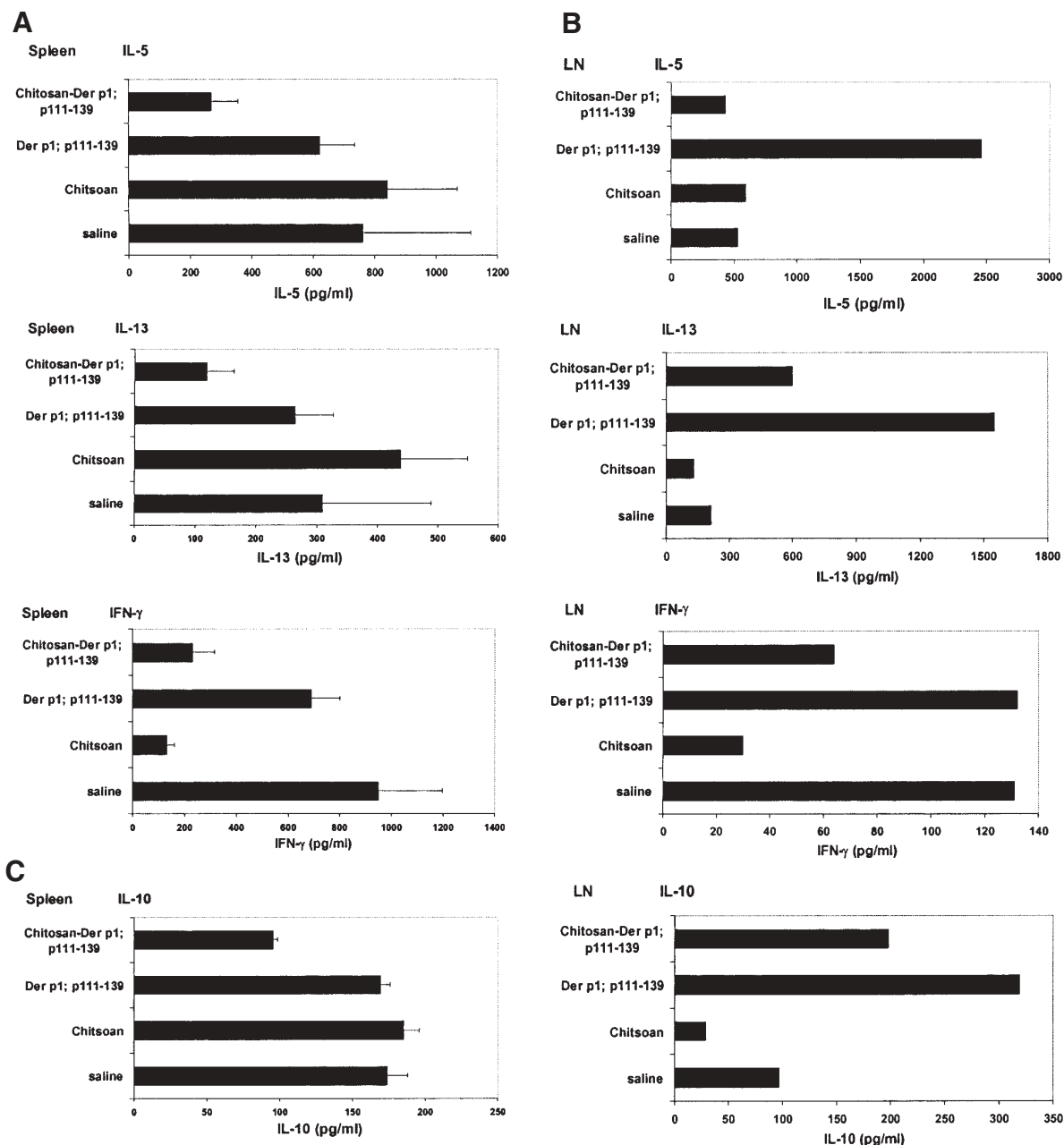


FIG 1. The induction of peripheral T-cell tolerance by the mucosal exposure to antigen is associated with a reduction in both T_H1 and T_H2 responses but also with an increase in IL-10 production. Mice tolerized by the intranasal instillation of peptide alone or peptide adsorbed to chitosan were sensitized and challenged with Der p 1. After 24 hours, cells isolated from spleen (A) or pooled bronchial lymph nodes (B) were restimulated in vitro with Der p 1 (10 μ g/mL); supernatants were collected after 72 hours, and the cytokines IL-5, IL-13, IL-10, and IFN- γ were measured by ELISA. Results are expressed for each group of mice as mean $\pm$ SEM ($n = 5$; Fig 1, A), as pooled data (Fig 1, B), or as a ratio of IL-10 to IL-5 production (C).

of peptide was sufficient to suppress T_H2 -mediated airway inflammatory responses to allergen challenge, we assessed the extent of eosinophil infiltration into the BALF. For this purpose, the lungs of Der p 1-challenged mice were lavaged 24 hours after antigen exposure. There was a significant ($P < .05$) reduction of 94% in the

extent of eosinophil recruitment into the lungs of mice pretreated intranasally with Der p 1;p111-139 peptide adsorbed to chitosan compared to the control mice treated with saline solution. No reductions in eosinophil recruitment were observed in mice given peptide alone (Fig 3, A).

The effect of intranasal peptide treatment on suppression of T_H2 activation and cytokine production in the airways

The T_H2 cytokines IL-4 and IL-5 are associated with eosinophil recruitment and survival in the lungs of allergic asthmatic individuals. We determined whether the loss of eosinophil recruitment was a direct effect of the reduction in levels of these cytokines in the lungs of tolerized mice after allergen challenge. Groups of mice receiving chitosan-adsorbed Der p 1;p111-139 peptide intranasally before systemic immunization showed significant reductions in BALF levels of 73% ($P < .01$) and 65% ($P < .05$) in IL-4 and IL-5, respectively, compared with control mice treated with saline solution. There was no reduction in IL-4 levels and there was a non-significant reduction of 48% in IL-5 levels in BALF obtained from mice treated with peptide alone. Intranasal administration of chitosan alone had no effect (Fig 3, B and C). Eosinophils are also a source of IL-5 in the airways. Reductions in the T_H2 cell cytokine IL-4, which initiates the influx of eosinophils into the airways through upregulation of adhesion molecules on endothelial cells, might more closely reflect changes in eosinophil levels in BALF.

The induction of sustained T-cell tolerance is preceded by transient activation marked by an increase in the levels of IFN- γ production

In several experimental models it has been reported that antigen-specific T-cell activation precedes the induction of peripheral tolerance. We investigated whether the differential effects of intranasally administered soluble peptide and peptide adsorbed to chitosan on the induction of tolerance was due to a failure to prime naive T cells or was instead due to differential effects on T-cell effector function. For this purpose, cervical lymph nodes draining the nasal cavity were removed either 48 or 96 hours after intranasal administration of peptide or peptide-chitosan and cells stimulated with increasing doses of Der p 1 p111-139 peptide. Peptide antigen-reactive T cells isolated from mice treated with peptide or peptide-chitosan were highly activated at 48 hours, showing dose-dependent increases in proliferation on in vitro stimulation with peptide. However, in mice given peptide-chitosan, reactive T cells exhibited 2- to 3-fold increases in IFN- γ production on stimulation with equimolar concentrations of peptide compared to peptide-treated mice. This was consistent over a dose range of peptide, indicating that it was not due to differences in TCR activation thresholds. Furthermore, at lower concentrations of peptide, IL-5 production was suppressed in T cells isolated from peptide-chitosan-treated mice (Fig 4, A). By 96 hours, T cells obtained from all groups of mice were nonresponsive to in vitro restimulation with peptide; there was a loss in both proliferative and cytokine responses (Fig 4, B). Chitosan itself failed to activate T cells.

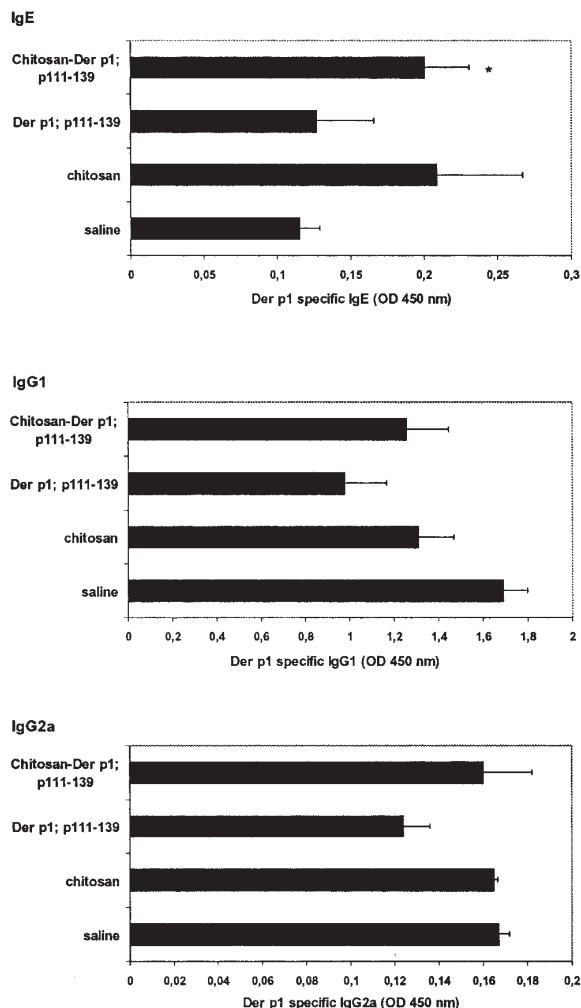


FIG 2. The induction of peripheral T-cell tolerance by mucosal peptide delivery has little effect on Der p 1 specific antibody levels in sera. Levels of Der p 1-specific IgE, IgG1, and IgG2a in sera were determined through use of ELISA. Serial dilutions of sera were set up in duplicate, and the results are presented as mean $\pm$ SEM ($n = 5$) for each group of mice; significant differences between treatment and control groups are indicated (* $P < .05$).

DISCUSSION

Here we have investigated, using a murine model of pulmonary inflammation, whether the intranasal administration of Der p 1;p111-139, a peptide covering an immunodominant T-cell epitope of Der p 1, could protect against the induction of T_H2 -mediated airway inflammatory responses to the Der p 1 allergen. A previous study demonstrated that intranasal administration of Der p 1 p111-139 peptide protected against T_H1 -mediated delayed-type hypersensitivity responses.¹⁷ We demonstrate that peptide alone was insufficient to prevent induction of T_H2 -mediated airway inflammatory responses unless it was adsorbed to chitosan, a compound that enhances uptake of antigen across the airway mucosa. The induction of tolerance in the antigen-specific T_H2

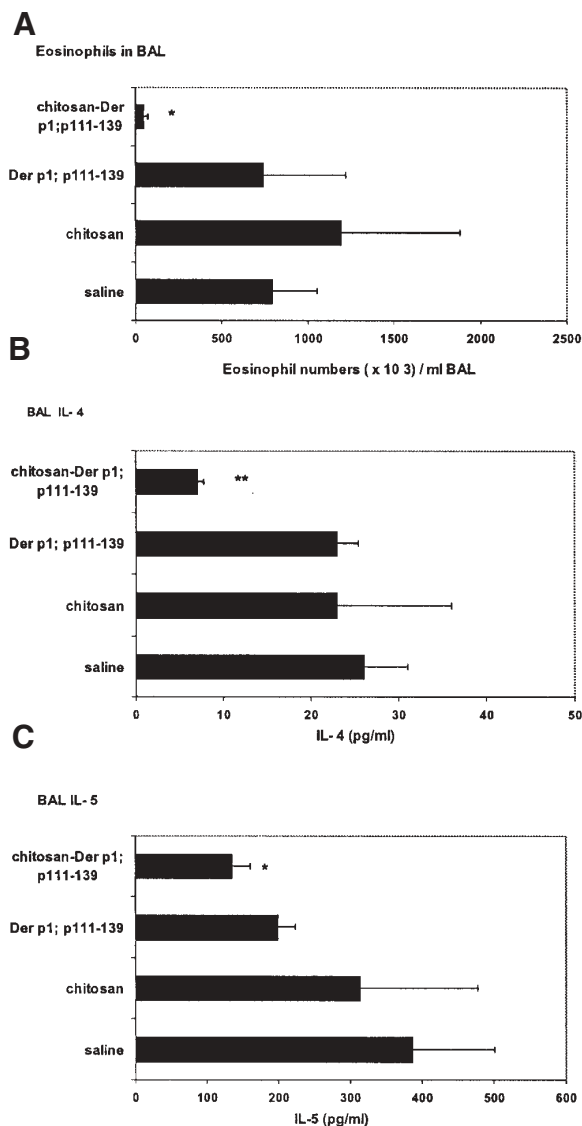


FIG 3. Effect of mucosal delivery of peptide on eosinophil recruitment and cytokine levels in BALF 24 hours after final challenge of the airways with Der p 1. Eosinophil levels in BALF were determined by Diff-Quik staining, and each number was calculated as a percentage of the total cell population per milliliter of BALF (**A**). Levels of the T_H2 cytokines IL-4 (**B**) and IL-5 (**C**) in BALF were measured through use of quantitative ELISA. These data are presented as means $\pm$ SEMs ($n = 5$) and significant differences between the treatment and control groups are indicated (* $P < .05$; ** $P < .01$).

population was preceded by transient increases in proliferation and IFN- γ production with suppression of IL-5.

Changes in the rate and dose of antigen delivery might affect the type of effector T-cell responses generated. The use of peptide adsorbed to chitosan, a compound widely used for controlled mucosal drug delivery,¹⁸ might influence the kinetics of APC activation in the mucosa.^{5,19} Changes in the density and affinity of MHC peptide complexes, the expression of costimulatory molecules and cytokines produced by the APCs during T-cell priming, differentially regulate the induction of T_H1 and T_H2 responses.^{20,21}

In vitro studies have demonstrated that qualitative and quantitative differences in antigen recognition might affect the affinity of interactions between the TCRs and their ligands and, consequently, the effector response generated. High-affinity TCR-MHC class II peptide interactions prime T_H1 responses, whereas low-affinity interactions prime T_H2 responses.²² The selective priming of T_H2 responses to inhalant allergens by pulmonary DCs²³ (pDCs) might therefore reflect not only differences in the cytokine milieu in the airways but also the low levels of antigen exposure and consequentially lower-affinity TCR-MHC class II-peptide interactions. Desensitization therapy, which involves the administration of increasing doses of allergen to allergic patients, is associated with both a shift in cytokine profile of allergen-reactive T cells from a T_H2 -like to a T_H1 -like response and a shift in the induction of T-cell tolerance.²⁴⁻²⁶ The transient increase in IFN- γ levels produced by hyperactive T cells isolated from peptide-chitosan-treated mice might be influential in the initial phase of tolerance induction. IFN- γ has been shown to play a protective role in the initial phase of tolerance induction in naive animals to inhalant antigen.²⁷

Mice that were given (intranasally) peptide adsorbed to chitosan exhibited a reduction in airway eosinophilia, associated with a fall in levels of the T_H2 cytokines IL-4 and IL-5 in BALF, which is suggestive of a loss of CD4⁺ effector T-cell recruitment and activation in the airways on allergen challenge. This correlated with a loss of IL-5, IL-13, and IFN- γ produced systemically and an increase in the ratio of IL-10 to IL-5 produced locally in the bronchial lymph nodes, on recall with the Der p 1 allergen. This suggests that attenuation of airway eosinophilia was a consequence of tolerance induction in the Der p 1-reactive effector T_H2 population rather than a T_H2/T_H0 to T_H1/T_H0 shift in cytokines.

Eosinophils are principal effector cells in the pathophysiology of allergic asthma.²⁸ The induction of airway inflammatory responses and recruitment of eosinophils into the airways after allergen exposure is largely initiated by CD4⁺ T_H2 cells through the production of IL-4 and IL-5. Both mast cells and eosinophils are also respective sources of these cytokines. The localized production of IL-4 in the airways has been shown to enhance expression of adhesion molecules such as VCAM-1 on endothelial cells, leading to the recruitment of eosinophils along a chemotactic gradient. IL-5 is a principal growth factor and chemoattractant for eosinophils, stimulating recruitment of progenitor cells from the bone marrow and stimulating their differentiation, maturation, and survival in the airways.^{29,30} A loss of antigen-specific priming of T_H2 effector responses in the bronchial lymph nodes and a reduction in IL-4 and IL-5 levels in the airways might account for attenuation of airway eosinophilia.³¹

In some patients, successful immunotherapy is associated with a shift in effector phenotype from a T_H2/T_H0 to a T_H1/T_H0 cytokine response, which might be achieved either by promoting T_H1 cytokines or by tolerizing T_H2 effector cells, both of which could shift the balance of cytokines. Studies in patients receiving bee venom

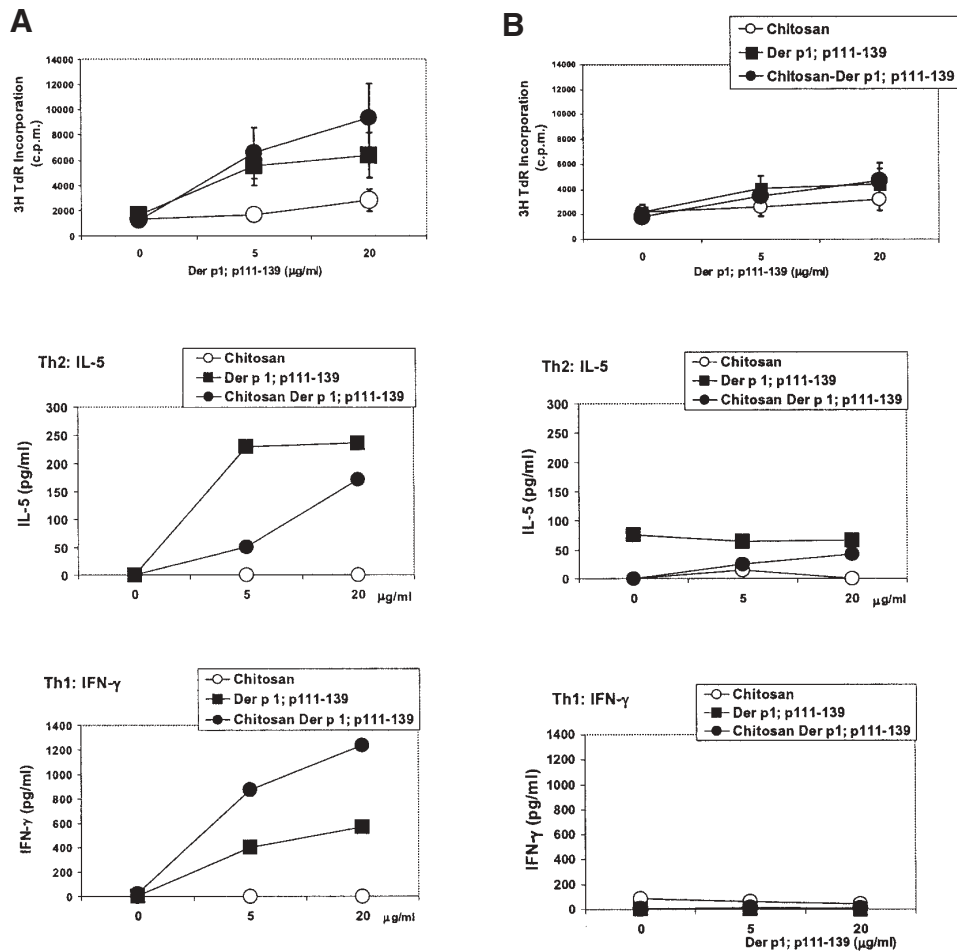


FIG 4. T-cell tolerance induced by mucosal delivery of antigen is preceded by transient activation. Groups of mice that were given (intranasally) peptide, peptide adsorbed to chitosan, or chitosan for 3 consecutive days were killed after 48 (**A**) or 96 (**B**) hours. Cervical lymph nodes were removed and cells were cultured in media or with Der p1; p111-139 peptide (at 5 or 20 μg/mL); proliferation was determined as 3H TdR incorporation after 72 hours. Culture supernatants were assayed for IL-5 and IFN-γ. These data are expressed as means ± SEMs (n = 5) for each treatment group.

immunotherapy demonstrate that suppression of antigen-specific T-cell proliferation and cytokine production is associated with T-cell anergy induction. Antigen-specific proliferation and T_H1 (IFN-γ) cytokine synthesis, but not T_H2 (IL-4, IL-5, IL-13) cytokine synthesis, could be restored by exogenous IL-2 and IL-15.²⁶ T-cell anergy was associated with increased levels of IL-10 production, primarily by antigen-specific $CD4^+CD25^+$ T cells in the periphery and subsequently by B cells and monocytes.³² IL-10 is an immunoregulatory cytokine that has been implicated in the reversal of T_H2 -mediated pathology, inhibiting both expansion and cytokine production by effector T_H2 cells and the recruitment of eosinophils into the airways. A potential role for IL-10 in mucosal tolerance induction was first suggested by studies that demonstrated an increase in IL-10 produced by antigen-specific T cells after the intranasal administration of an immunodominant epitope of myelin basic protein.³³ However, until now a role for IL-10-producing T regulatory cells in the induction and maintenance of peripheral tolerance to

inhalant aeroallergen had not been shown. The immunoregulatory cytokine IL-10, produced by pDCs after exposure of the airways to soluble antigen, might play a role in downregulating airway inflammatory responses. Production of IL-10, in conjunction with the expression of a mature phenotype by pDCs on migration to the bronchial lymph nodes where antigen is presented to naive T cells, might be instrumental in the induction and maintenance of tolerance to environmental antigens encountered in the respiratory mucosa. pDCs obtained after mucosal exposure to soluble antigen from wild-type mice, but not from IL-10 knockout mice, were shown to adoptively transfer tolerance to naive mice and, in vitro, to prime IL-10-producing $CD4^+$ Tr1 cells.³⁴ IL-10 is now recognized as a growth factor for a population of $CD4^+CD25^+$ Tr1 cells,³⁵ originally identified in the thymus, and is now thought to play a critical role in maintaining peripheral tolerance.³⁶ There is considerable evidence that pDCs play a critical role in the induction of tolerance to inhalant antigen and in the generation of Tr1

cells. Our data suggest that these antigen-specific Tr1 cells might play an important role in maintaining tolerance through production of IL-10 in lymph nodes draining the airways after antigen challenge. IL-10 induces a tolerogenic phenotype in DCs, which correlated with a failure to undergo maturation and upregulation of the costimulatory molecules CD80, CD86, and CD40.³⁷ The production of IL-10 might therefore account for the observed bystander suppression with a loss of responses not only to the Der p 1:p111-139 epitope but also to minor T-cell epitopes within Der p 1.

In conclusion, we demonstrate that the mode of peptide delivery to the airway mucosa might be fundamental to the induction of sustained T_H2 tolerance to inhaled allergen and reductions in airway eosinophilia. The increase in IFN- γ production and suppression of IL-5 during the initial activation phase and the production of IL-10 in the bronchial lymph nodes following antigen exposure might be instrumental in maintaining a tolerogenic state. Within the limitations of the model system used, these studies give some indication of parameters that might be of importance in designing immunotherapeutic approaches for allergy.

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