

Hypersensitivity reactions after respiratory sensitization: Effect of intranasal peptides containing T-cell epitopes

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Background: The intranasal administration of peptides containing T-cell epitopes has been shown to inhibit T-cell and antibody responses of mice injected with allergen, but responses to respiratory sensitization might be regulated differently. **Objective:** This study was designed to examine the effect of intranasal peptide on antigen-induced lung inflammatory responses and delayed hypersensitivity after sensitization by the respiratory mucosa or without sensitization.

Methods: Mice were treated with an intranasal tolerizing regimen of a peptide containing the major T-cell epitope of Der p 1. Delayed hypersensitivity and lung inflammation to challenge with Der p 1 was measured either without further treatment or after sensitization induced by means of the intranasal administration of Der p 1 with a mutated enterotoxin adjuvant. Lung inflammatory responses were examined by means of lavage and histologic section, and delayed hypersensitivity responses were measured on the basis of ear swelling.

Results: Delayed hypersensitivity reactions were induced in mice treated with intranasal peptide, and large reactions were found in mice given intranasal peptide and sensitized with intranasal Der p 1 and adjuvant. Mice pretreated with peptide and sensitized with Der p 1 had an increased lymphocytic infiltration after allergen-specific challenge, as measured by means of bronchoalveolar lavage and shown histologically. These hypersensitivity results are in contrast to previous data that show tolerance to injected antigen.

Conclusions: Although the intranasal administration of a peptide containing a T-cell epitope markedly inhibits responses to sensitization produced by the injection of allergen, the peptide induces immune responses and increases hypersensitivity to respiratory sensitization. (*J Allergy Clin Immunol* 2002;110:610-6.)

Key words: Intranasal peptide, respiratory sensitization, hypersensitivity, inflammation, Der p 1, mouse

Abbreviation used

HSA: Human serum albumin

The intranasal administration of antigen induces a type of immunologic tolerance,¹⁻⁵ which might have the potential to downregulate allergic sensitization. Short regimens of ovalbumin²⁻⁴ or the birch allergen Bet v 1⁵ have been shown to inhibit immune responses to the injection of antigen and the inflammatory responses found after subsequent respiratory challenge.^{4,5} In the case of Bet v 1, the inhibition of IgE antibody was accompanied by a decreased production of IgG and T_H1 and T_H2 cytokine levels, including IL-10. Transforming growth factor β was increased, suggesting a regulatory mechanism. For sensitization with ovalbumin, selective inhibition of IgE has been found^{2,4} without the production of transforming growth factor β .⁴ In one study IFN- γ was increased,² whereas in another study all cytokines were reduced.⁴ Delayed hypersensitivity can also be inhibited.³ Antigen-specific variation in the effect of intranasal peptide was noted by Wiedermann et al,⁶ who showed that a regimen that inhibited responses to Bet v 1 did not affect responses to ovalbumin. The variations might occur because the outcome of the intranasal antigen administration is balance between activation and inhibitory events. This can readily be observed during chronic exposure,¹ in which a variable transient IgE response and priming for IgG is produced. The related phenomenon of inhalation tolerance similarly inhibits IgE production while activating IFN- γ -producing T cells.⁷

After studies with autoimmunity,⁸ the inhibitory ability of T-cell epitopes presented as synthetic peptides or hypoallergenic molecules has been examined.⁹⁻¹⁵ The IgE response to allergen injected in alum can be inhibited, as shown with the dominant epitope of *Dermatophagoides pteronyssinus*,¹² long peptides of honeybee phospholipase A2,¹³ and recombinant polypeptide fragments of Bet v 1.¹⁵ The fragments of Bet v 1 inhibited all T- and B-cell responses and an inflammatory response to respiratory challenge. The phospholipase A2 peptides inhibited T_H2 responses with an increase in IgG2a antibody and the ratio of T_H1/T_H2 cytokines. The Der p 1 peptide inhibited IgE production and delayed hypersensitivity but did not affect serum IgG1 or IgG2a titers.¹² It was preceded by an

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activation phase in which T cells in the lymph node were primed to produce IL-2, IFN- γ , and GM-CSF.¹⁰ These T-cell responses could be restimulated by means of intranasal peptide at a time when the mice were unable to respond to antigen injected in adjuvant. Similar activation phenomena have been reported for oral tolerance¹⁶ and for intranasal administration of a peptide from the mycobacterial hsp65 protein.¹⁷

The ability of T cells from mice treated with intranasal peptide to reactivate on further intranasal challenge suggested the need to study sensitization induced through the respiratory mucosa. When the sensitization was induced intranasally with an enterotoxin adjuvant, it was found that pretreatment with peptide did not inhibit IgE and IgG responses, but it partially reduced IL-4 and IL-13 responses.¹² Bronchoalveolar lavage of the mice after intranasal challenge showed an increased recovery of lymphocytes from the peptide-treated mice. This inflammatory effect has now been investigated further. The results here show that intranasal peptide specifically increased inflammatory responses in the lung and induced delayed hypersensitivity. The reactions demonstrate that although antigen presentation in the respiratory mucosa induces a form of tolerance,¹⁸ it might also induce unwanted hypersensitivity.

METHODS

Mice

Specific pathogen-free C57BL/6 mice (6-8 weeks old) were obtained from the Animal Resource Centre (Murdoch, Australia).

Antigens and peptides

Der p 1 was isolated from a 20% solution of spent mite media (a gift from CSL-Ltd, Melbourne, Australia) dissolved in PBS by means of affinity chromatography with mAb 4C1 provided by Dr M. D. Chapman (Indoor Biotechnologies, Charlottesville, Va). Human serum albumin (HSA) was purchased from Sigma (St Louis, Mo). The peptide representing residues 114-128 of Der p 1.0101¹⁹ (SNYCQIYPPNANKIR) was synthesized by Chiron Mimotopes (Clayton, Australia) with a free C-terminus and was verified by means of mass spectrometry by the manufacturer.

Sensitization and intranasal administration of peptide

Mice were treated on 5 consecutive days with 100 μ g of peptide in 20 μ L of PBS. The peptide was administered dropwise to the nares with light anesthesia. The peptide represented the dominant T-cell epitope for H-2^b mice found between amino acids 114-126 of the allergen Der p 1.0101.¹⁹ When required, sensitization was performed 10 days after the last dose of peptide. Mice were sensitized with 100 μ g of Der p 1 or HSA with 10 μ g of mutated *Escherichia coli* heat-labile enterotoxin adjuvant in 20 μ L of PBS. The solution was applied dropwise to the nares of lightly anesthetized mice. The production of the adjuvant, which had a deletion of residues 192-194 of the A chain, has been described.²⁰ It has very little toxicity and enhanced adjuvant activity over the natural molecule. Mice were challenged for lung inflammation or delayed hypersensitivity 4 weeks after administering the sensitizing antigen. The same timing was used for mice that were only treated with peptide, except that they did not receive the antigen. For 2 applications of intranasal peptide, a second regimen of 5 daily doses of peptide was begun 10

days after the end of the first application, and the delayed hypersensitivity was measured 4 weeks after the beginning of a second application. This kept the timing the same for all experiments, as shown in the summary of the protocols (Table I).

Delayed hypersensitivity

Mice were challenged 4 weeks after sensitization with an intradermal injection of 10 μ L of 1 mg/mL Der p 1 or HSA in PBS into the ventral side of the pinnae of left ears. After 24 hours, the thickness of both ears was measured with an engineer's micrometer, and the difference was used as a measure of delayed hypersensitivity. The swelling, expressed in units of 10⁻² mm, was compared with the swelling found for nonsensitized mice.

Lung inflammation

Mice were challenged 4 weeks after sensitization by means of intranasal administration of 100 μ g of Der p 1 or HSA in 20 μ L of PBS on 3 consecutive days. The following day, the mice were killed and immediately perfused by injecting PBS through the left atrium. The bronchus to the right lobe was clamped, and the lobe was removed and placed in 10% saline-buffered formalin. An excision was made between the cartilaginous rings of the trachea, and a 2-mm-diameter polyethylene cannula connected to a 23-gauge needle was inserted. The left lobe underwent lavage 3 times with 300 μ L of PBS/2% BSA. For differential counts, cytospin preparations of 5 $\times$ 10⁴ cells in 100 μ L were made with a cytocentrifuge (Elliot Shandon, Pittsburgh, Pa). Cells were air dried for 30 minutes and stained with Diff Quick (Lab Aids, Narrabeen, Australia), according to the manufacturer's instructions. Paraffin-embedded tissue sections were stained with hematoxylin and eosin by using standard procedures.

RESULTS

Mice were given 5 daily doses of the Der p 1 peptide to examine the effect of intranasal peptide treatment on respiratory sensitization, and after 10 days, they were sensitized by means of an intranasal administration of Der p 1 in mutant toxin. After a further 4 weeks, the mice were challenged with intranasal administration of 100 μ g of Der p 1 on 3 consecutive days, and bronchoalveolar lavage fluid and lung histologic specimens were examined the next day. The inflammation was compared with the responses of groups administered PBS instead of peptide or mice that had not been sensitized with Der p 1 and adjuvant. Table II and Figs 1 and 2 show the results of one of 3 experiments. The bronchoalveolar lavage and histology results show that the inflammation primarily consisted of mononuclear cells with some eosinophils, but the latter were variable. The eosinophils were more frequent and consistent in the HSA-sensitized mice (see below). The mice pretreated with peptide showed an increased inflammatory response characterized by the increased number of lymphocytic cells in the lavage fluid and histologic sections. The low-power view in Fig 1 illustrates the general increase in cells. The inflammation was primarily perivascular in the mice sensitized with Der p 1 without the pretreatment with peptide. Mice that had been pretreated with peptide not only had an increased number of inflammatory cells, but the inflammation frequently extended to the airway epithelium (Fig 2). Mice given only the peptide did not show an increase in lymphocytic cells in the lavage fluid. Mice were treat-

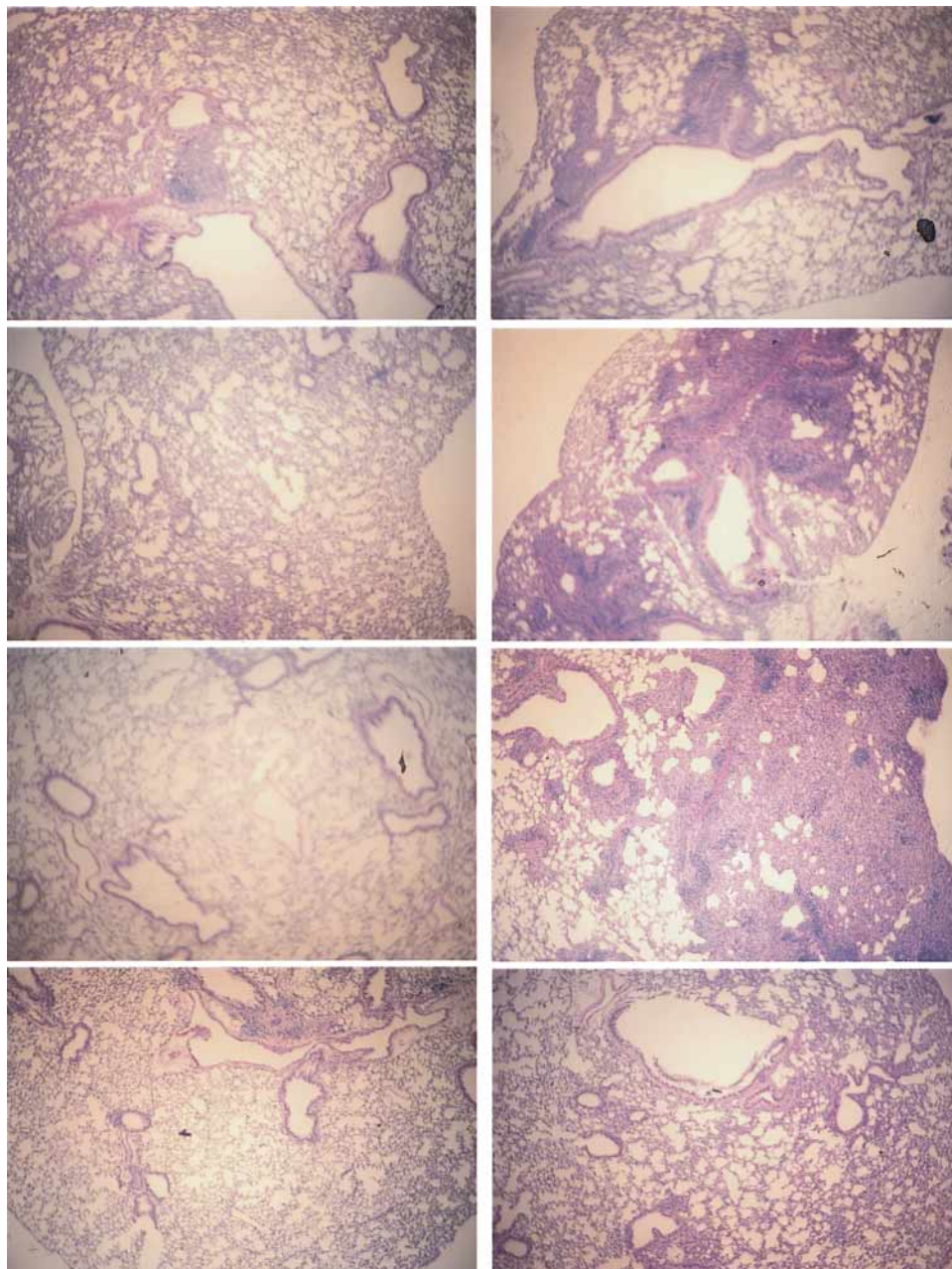


FIG 1. Lung inflammatory response of mice pretreated or not pretreated with peptide. Mice were pretreated with PBS (*left column*) or peptide 114-128 (*right column*), sensitized intranasally with Der p 1, and, after 4 weeks, challenged with Der p 1. The photomicrographs show a low-power view (original magnification 40 $\times$) of hematoxylin and eosin-stained sections from 4 mice for each regimen.

ed with the Der p 1 peptide 114-128 and sensitized and challenged with HSA by using the regimen used for Der p 1 to test for specificity. No increase in the inflammatory response was found (Table I).

Mice were treated with the same regimens used for respiratory challenge to examine delayed hypersensitivity but, at week 4, were injected with antigen in the pinnae of

TABLE I. Sensitization protocols

Pretreatment	Intranasal peptides (100 μ g) on days 1-5
Sensitization	Intranasal antigen in adjuvant day 15 or second course of peptides on days 15-19
Challenge	Intranasal antigen on days 44-46 for lung inflammation or ear injection on day 44 for delayed hypersensitivity

the ear. The swelling measured after 1 day showed that mice sensitized with intranasal Der p 1 and the adjuvant produced swelling responses that were of similar magnitude to that previously reported for mice immunized with Der p 1¹⁰ in CFA (Table III). The histology of the ear sections showed the mononuclear cell infiltration and edema typical of murine delayed hypersensitivity reactions. Mice that had received the intranasal peptide before treatment but without further sensitization showed significant swelling reactions. Mice that had been pretreated with the peptide before the sensitization with Der p 1 tended to have larger swelling reactions than mice that had been treated with PBS before the sensitization, but no synergistic effect was evident. Control experiments showed that pretreatment with the Der p 1 peptide did not increase the swelling response of mice sensitized and challenged with HSA, and mice given Der p 1 peptide alone did not produce swelling responses when challenged with HSA. Because the peptide treatment itself induced delayed hypersensitivity, the effect of a peptide pretreatment on the response to the peptide was examined. The regimen was the same as for previous experiments, except that instead of sensitization with Der p 1 at day 15, the mice were given a further 5 doses of peptide. Mice given the first peptide treatment (day 1-5) had significantly higher swelling than the control mice, and mice given only the second peptide (day 15-19) treatment had even higher responses, which is consistent with the more recent sensitization. Mice given the first and second treatments had the same responses as mice given the second sensitizing course of peptide. Mice that had been pretreated with one course of peptide therefore produced a delayed hypersensitivity response to a second course.

DISCUSSION

The intranasal administration of Der p 1 peptide has been shown to profoundly inhibit IgE antibody responses of mice injected with Der p 1 in alum¹² and to inhibit delayed hypersensitivity and IL-2, IFN- γ , and IL-5 responses of mice immunized with the allergen in CFA.^{11,12} This concurs with studies of others with intranasal administration of allergen¹⁻⁵ or peptides.¹³⁻¹⁵ In contrast, when the sensitization was performed intranasally, neither the IgE and IgG antibody responses nor the IL-2, IFN- γ , IL-10, and IL-5 responses were affected.¹² However, a 50% decrease in IL-4 and IL-13 release was found. Thus mice that could only produce minimal responses to the injection of antigen responded almost normally to a different form of antigen presentation. The altered response was not due to the enterotoxin adjuvant because the responses of mice treated with the adjuvant and immunized with allergen in alum were still inhibited. The results presented here show that the mice treated with peptide and sensitized intranasally had increased delayed hypersensitivity and antigen-specific lung inflammatory responses. These responses could be related to the reduced production of T_H2 cytokine, either by cause or effect, although the ability of the lymph node

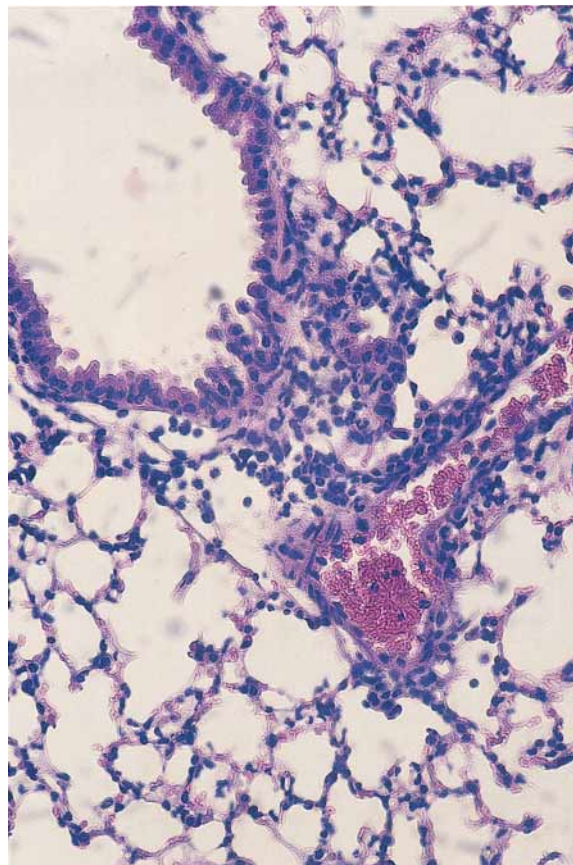


FIG 2. Lung inflammation of peptide-pretreated mice: section of lung from a mouse pretreated with peptide 114-128, sensitized intranasally with Der p 1, and, after 4 weeks, challenged with Der p 1. A lymphocytic perivascular and airway infiltrate can be seen (original magnification 160 $\times$).

cells to produce IFN- γ has been shown to be unaffected.

The magnitude of the delayed hypersensitivity, IgG, and IgE responses induced by the respiratory sensitization were almost identical to the responses induced by means of immunization in alum or CFA.¹² They are midrange responses; antibody can be boosted, and the swelling of the delayed hypersensitivity can range from 2 to greater than 30 units. The time course for the systemic and respiratory sensitization was also very similar. The dose of responses for the respiratory sensitization with mutant toxin has been examined (A.G.J., unpublished observations). The lower dose of 10 μ g of Der p 1 induced low antibody titers, and half the amount of mutant toxin was not stimulatory. The respiratory sensitization was therefore performed under titratable conditions. The amount of antigen reaching the lung from intranasal installation has been measured, and only a few micrograms reach the lung, but the antigen persists in an immunologically active form in the draining lymph nodes for at least a day.²¹

The information described in this article was sought because treatments that increase lung inflammation might have limitations for immunotherapy. The

TABLE II. The effect of peptide treatment on cells from bronchoalveolar lavage fluid

Pretreatment	Sensitization	Macrophages, cells/mL (10^{-3})	Lymphocytes, cells/mL (10^{-3})	Neutrophil, cells/mL (10^{-3})	Eosinophil, cells/mL (10^{-3})
PBS	Der p 1	628 $\pm$ 213	41 $\pm$ 24	10 $\pm$ 6	1 $\pm$ 1
p114-128	Der p 1	487 $\pm$ 162	145 $\pm$ 48*	79 $\pm$ 52	42 $\pm$ 40
No treatment	—	748 $\pm$ 193	2 $\pm$ 1	1 $\pm$ 0.5	—
p114-128	—	568 $\pm$ 215	2 $\pm$ 0.7	2 $\pm$ 0.8	—
PBS	HSA	674 $\pm$ 195	63 $\pm$ 60	286 $\pm$ 133	99 $\pm$ 43
p114-128	HSA	961 $\pm$ 304	25 $\pm$ 25	297 $\pm$ 137	65 $\pm$ 18
No treatment	—	839 $\pm$ 485	0	24 $\pm$ 4	4 $\pm$ 1

Mice were treated on 5 consecutive days with either PBS or 100 μ g of peptide administered intranasally and sensitized 10 days later intranasally with 100 μ g of Der p 1 with mutated enterotoxin adjuvant. After 4 weeks, the mice were challenged with 100 μ g of Der p 1 per day for 3 consecutive days, and bronchoalveolar lavage was performed the next day. As a control, mice were sensitized with HSA. Data represent the mean $\pm$ SD of each group. Peptide treatment induced a significant increase (*) in lymphocyte cell numbers ($P < .05$).

TABLE III. Effect of peptide treatment on delayed-type hypersensitivity

Pretreatment	Sensitization	Challenge	Ear swelling (10^{-2} mm)
PBS	Der p 1	Der p 1	15.8 $\pm$ 6.2
p114-128	Der p 1	Der p 1	23.6 $\pm$ 5.2
p114-128	—	Der p 1	9.7 $\pm$ 4.2*
No treatment	—	Der p 1	3.5 $\pm$ 3.1
PBS	HSA	HSA	16.2 $\pm$ 2.9
p114-128	HSA	HSA	15.6 $\pm$ 3.0
p114-128	—	HSA	4.6 $\pm$ 1.9
No treatment	—	HSA	3.7 $\pm$ 3.0
PBS	p114-126	Der p 1	11.4 $\pm$ 2.9
p114-126	PBS	Der p 1	6.9 $\pm$ 1.2*
p114-126	p114-126	Der p 1	12.4 $\pm$ 1.9†
PBS	PBS	Der p 1	5.1 $\pm$ 1.2‡

Mice were treated on 5 consecutive days with either PBS or 100 μ g of p114-128 administered intranasally and sensitized 10 days later with 100 μ g of Der p 1 administered intranasally with mutated enterotoxin adjuvant. After 4 weeks, the mice were challenged in the ear with 10 μ g of Der p 1 or HSA as a control, and the ear-swelling responses were measured after 24 hours. The data represent the mean $\pm$ SD. p114-128 did not inhibit responses to intranasal sensitization but did induce a significant response (*) without sensitization compared with PBS ($P < .05$, Student *t* test). Double p114-128 administration stimulated response († $P < .01$).

‡Negative control for double-peptide administration.

intranasal administration of peptides has been viewed primarily as an energizing treatment that might have therapeutic or prophylactic application. The histologic sections here show the potential for increased cellular infiltration and damage of the airway epithelium. The cellular infiltration in the lungs of mice that had only been sensitized with Der p 1 was primarily perivascular, which corresponded with the low numbers of lymphocytes in the lavage fluid. Mice pretreated with intranasal peptide show inflammation extending to the airways and had lymphocytic cells in the lavage fluid. The mice given the peptide treatment alone did not show an inflammatory response to respiratory challenge but did show delayed hypersensitivity when challenged in the ear. The delayed-type hypersensitivity reactions were specific in that they could be elicited with Der p 1 but not HSA. The ability to detect increased delayed hypersensitivity and not an increase in lung inflammation responses after peptide treatment might warrant further investigation, but it could reflect the nature of the ear-swelling test. Only a few active cells are required,²² and the swelling, which is dependent on cytokine production rather than the accu-

mulation of cells, is easier to elicit in the ear than in many other sites.²³ The finding that peptide treatment itself induced delayed hypersensitivity is in keeping with previous results,¹⁰ which showed a transient activation of T cells. It was also possible to reactivate the cells with the intranasal peptide. The results here show that a second course of intranasal peptide was able to reactivate the delayed hypersensitivity at a time when delayed hypersensitivity cannot be induced with antigen in CFA.¹²

Although the focus of this study was on the response to intranasal peptides, it further characterizes the effect of the mutant enterotoxin adjuvant. The Arg 192-Ile 194 deletion produced in the A chain of the heat-labile enterotoxin prevents a critical posttranslational cleavage step, and the adjuvant is almost devoid of ADP-ribosyltransferase activity and toxicity.²⁰ It retains very strong adjuvant activity, which has now been demonstrated for several antigens. It was originally shown to be an adjuvant for IgG, IgE, and IgA antibody responses,²⁰ and the responses to Der p 1 show that it is a mixed T_H1/T_H2 adjuvant inducing high titers of IgE, IgG1, and IgG2a and T cells that produce IL-2, IL-4, IL-5, IL-10, IL-13,

and IFN- γ .¹² Long-term delayed hypersensitivity and cell-mediated immunity to varicella virus has been described,²⁴ as has hypersensitivity to the protein antigens described here. The lung inflammation induced by Der p 1 and HSA shows that although the T cells of mice sensitized with the adjuvant are mixed T_H1/T_H2 cytokine producers and the mice produce high titers of IgE, the infiltration is mononuclear, with a few eosinophils. However, the response to HSA was more eosinophilic.

Adjuvant was used for sensitization because, like the intranasal administration of other proteins,¹ the intranasal administration of Der p 1 to high-responding C57BL/6 and BALB/c-H-2^b mice induces low IgE responses, which are sporadic and decline with or without repeated doses of allergen (unpublished). The intranasal application of the Der p 1 peptide itself, however, does, as described before, induce T-cell responses, and these responses can be reactivated by repeated doses of peptide. Although it would be of interest to examine a mouse model of respiratory sensitization with a more consistent eosinophilia, this has only been accomplished for complex extracts, such as *Aspergillus* and latex antigens, which might have their own adjuvant activity.^{25,26} However, it should be noted that T cells from the lungs of asthmatic patients have very mixed cytokine T_H1/T_H2 profiles,²⁷ and asthmatic patients do not necessarily have a prominent airway eosinophilia.²⁸ A recent study has proposed that patients with severe asthma can be divided into eosinophil-positive and eosinophil-negative groups, with each group having the same level of IgE and atopy.²⁹ The events that trigger the induction of sensitization in human subjects are not known, but the difficulty in sensitizing mice indicate that an adjuvant activity is required. Although there is no evidence for the role of exotoxins, it should be noted that the ADP-ribotransferases used here are powerful mucosal adjuvants, and they are extremely abundant, being found in common respiratory infections, including *Pertussis* species, staphylococci, and *Pseudomonas* species.³⁰

The combined data from the responses induced with intranasal peptide¹⁰ and intranasal Der p 1 and adjuvant suggest that the tolerizing effect of intranasal peptide might be limited to systemic immunization. The intranasal administration of peptide can induce proliferation of T cells in all lymphoid tissues.¹⁴ However, it might deliver activating signals to the cells in the lymph nodes, draining the respiratory system, where danger signals are encountered, and downregulating cells exposed to peptide at the distant sites, the populations that would respond to injected antigen. The notion that activated T cells migrate or survive best in their tissue of sensitization has also been proposed,³¹ but although T cells in the lungs have a unique pattern of adhesion molecules and chemokine receptors,³² direct evidence is lacking. It is unlikely that the mechanism would only involve a change in trafficking patterns because the Der p 1 peptides induce a suppressive mechanism in which the administration of a peptide containing a single T-cell epitope inhibits responses to other epitopes on the molecule.^{9,11}

and responses to coadministered antigens.¹² However, it is possible that the anergic cells in the nonrespiratory compartment mediate the suppression during the responses to injected antigen.³³ A different possibility is that cells activated by the first exposure remain in the respiratory population and produce cytokines that prevent the induction of tolerance.³⁴ As shown for viral infection, activated T cells persist in the lung for at least months, and on reinfection, cells from the draining nodes quickly expand and move to the lung.^{35,36} The α E β 7-positive T cells that persist in the airway epithelium might also have a role, although their function is unknown.³⁷

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