

Inhibition of mucosal and systemic T_H2-type immune responses by intranasal peptides containing a dominant T cell epitope of the allergen Der p 1

Andrew G. Jarnicki, Takao Tsuji¹ and Wayne R. Thomas

University of Western Australia Centre for Child Health Research and Department of Microbiology, TVW Telethon Institute for Child Health Research, PO Box 855, West Perth, WA 6872, Australia
¹Department of Microbiology, Fujita Health University, School of Medicine Toyoake, Aichi, Japan

Keywords: inhibition, Der p 1, IgE, peptides, intranasal

Abstract

Although the intranasal administration of peptides containing T cell epitopes has been shown to be a potent method of inhibiting responses to the allergen Der p 1, the experiments to date have concentrated on their ability to regulate immune responses to the injection of antigen in a T_H1-type adjuvant. Their ability to regulate responses to a T_H2-type immunization and to sensitization via the respiratory mucosa has not been examined. Here it is shown that peptide used in doses required to block delayed-type hypersensitivity can also readily inhibit IgE responses to Der p 1 injected in alum. To examine responses induced in the respiratory mucosa, mice pretreated with intranasal peptide were sensitized with an intranasal dose of Der p 1 in conjunction with a mutated enterotoxin adjuvant. Intranasal peptide even in very high doses did not reduce IgE titers, but the ability of cells from the draining lymph nodes to release IL-4 and IL-13 but not IL-2, IL-5, IL-10 or IFN- γ was reduced. These are the first reports on the effect of intranasal peptides containing T cell epitopes on IgE in T_H2 immunization and on responses to respiratory immunization. Thus the effect of the peptide-induced mucosal tolerance differs depending on the type of immunization used for sensitization, but the potential to inhibit T_H2 responses and responses to respiratory sensitization as well as T_H1 responses has been demonstrated.

Introduction

The intranasal administration of proteins or peptides containing T cell epitopes can be a potent inhibitor of immune responses. This phenomena has been extensively studied in models of autoimmunity where the induction of disease and T_H1-type responses can be markedly reduced (1). It has sometimes but not always been associated with increased T_H2 cytokine production (2–5). Studies with the house dust mite allergen Der p 1 have shown that responses to exogenous antigens can also be inhibited by intranasal peptides and have raised the possibility that peptides can be used to treat or prevent allergic disease. The initial studies with the allergen reported that a peptide containing an individual T cell epitope could block the induction of T cell responses to all the epitopes on the allergen as measured by IL-2 release in *in vitro* T cell stimulation assays (6). The peptides could also inhibit the induction of delayed-type hypersensitivity (DTH)

showing their ability to inhibit responses in the whole animal (7). Although these experiments showed that T_H1-responses were inhibited by a mechanism involving intramolecular suppression, the regulatory effect on IgE in a T_H2-biased response and responses to respiratory exposure have not been reported. This question needs to be resolved because although it has been reported that intranasal peptides did not affect IgE titers, the responses examined were T_H1-biased responses induced by the injection of Der p 1 in complete Freund's adjuvant (CFA) (7). The regulatory mechanism in T_H2-biased responses, however, can be different. For example, any inhibitory effect on T_H2 responses could be equally matched by a decrease in cross-regulatory T_H1 cytokines. It is equally necessary to examine responses induced by sensitization via the respiratory mucosa, particularly since it has been found that a secondary intranasal challenge with

peptide can re-stimulate T cell responses in the draining lymph nodes and spleen at a time when the mice are tolerant to injected antigen (8). Similar responses have been found after multiple feeding (9).

Here we report that the intranasal administration of peptides containing the dominant T cell epitope of Der p 1 can markedly and selectively decrease IgE antibody isotype produced in response to the allergen injected in alum, a regimen which stimulates T_H2 responses. The effect on sensitization by the respiratory route was different where the intranasal peptides did not decrease IgE but reduced the production of the T_H2 cytokines IL-4 and IL-13, but not the T_H1 cytokines IL-2 or IFN- γ or the regulatory IL-10.

Methods

Mice

C57BL/6J mice (6–8 weeks old) were obtained from the Animal Resource Centre (Murdoch, Australia) and kept under specific pathogen free conditions.

Antigens and peptides

Der p 1 was isolated from a 20% spent mite medium dissolved in PBS (gift from CSL, Melbourne, Australia) by affinity chromatography using the mAb 4C1 provided by Professor M. D. Chapman (University of Virginia, Charlottesville, VA). Ovalbumin (OVA) crystalline grade V was purchased from Sigma (St Louis, MO). Peptides were synthesized by Chiron Mimotopes (Clayton, Australia) with a free C-terminus, and analyzed by the manufacturer for purity and yield by reverse-phase HPLC and verified by mass spectrometry (mol. wt = 2151.4 for p114–128).

Immunization and intranasal administration

Mice were immunized either i.p. with 10 μ g of antigen adsorbed to 10 mg aluminum hydroxide gel in 100 μ l PBS or s.c. at the base of the tail with 100 μ g antigen in 100 μ l of PBS emulsified with 100 μ l CFA (Difco, Detroit, MI). For intranasal administration, the peptides and antigens were dissolved in PBS and 20 μ l administered to the nares of lightly anaesthetized mice with a micropipette. Previous studies have used a peptide consisting of residues 110–131 of Der p 1 but most studies here were performed with peptide 114–128 (SNYCQIYPPNANKIR) which spans the minimal major CD4 H-2^b epitope between residues 118–126 (authors' unpublished observations).

Respiratory sensitization was produced by the intranasal administration of 100 μ g of Der p 1 with 10 μ g of a mutated subunit of *Escherichia coli* heat-labile enterotoxin in 20 μ l of PBS. In some experiments the responses were boosted by the same dose of Der p 1 and mutated toxin. The mutant toxin has been shown to have very little toxicity and enhanced adjuvant activity without inducing cAMP (10). It was a deletion mutant lacking R192, T193 and I194 constructed by PCR from a *HindIII*–*EcoRI* fragment of the LT-A subunit gene. The mutant plasmid was named as pTSU135. After pTSU135 was transformed into the *E. coli* MV1184 strain, the mutant LT protein was purified with immobilized D-galactose-affinity column chromatography (10).

T cell assays

Lymph node cells were assayed for antigen-induced cytokine production 10 days after immunization. For mice immunized with antigen in CFA the inguinal and periaortic lymph nodes were used and for respiratory immunization the superficial cervical lymph nodes. The nodes were pressed through a stainless steel mesh, washed and cultured at 5×10^5 cells/well in 96-well round-bottomed 96 microtiter trays (Nunc, Roskilde, Denmark) in 200 μ l of DMEM medium (Gibco, Grand Island, NY) supplemented with 2% FCS (CSL, Melbourne, Australia), 50 mM L-glutamine (Gibco) and 50 μ M of 2-mercaptoethanol (BDH supplies, Poole, UK) and 20 mM HEPES (ICN Biochemicals Co., OH). Antigen was added to the cultures at various concentrations and the cells incubated at 37°C in a 5% CO₂ atmosphere for 24 or 48 h. Supernatants were stored at –20°C until required. IL-2 was assayed by measuring the [³H]thymidine incorporation of CTLL-2 indicator cells (7), and IL-4, -5, -10 and -13 and IFN- γ were measured by sandwich ELISA. Maxisorb plates (Nunc) were coated with a monoclonal capture antibody by incubating at 4°C overnight with antibody diluted to the suppliers instructions in 0.1 M Na₂HPO₄, pH 9.0. The plates were washed 5 times in 0.05% Tween 20 in PBS and blocked with 1% BSA in PBS for 1 h. The plates were washed as above, and 50 μ l of the samples to be assayed was added and incubated for 2 h at room temperature with shaking before a further 5 washings and the addition of 50 μ l of biotinylated detection antibody diluted in PBS containing 0.05% Tween 20 and 1% BSA. After 1 h the plates were washed 5 times and incubated with 100 μ l of streptavidin–multi-horseradish peroxidase (CLB, Amsterdam, The Netherlands) for 30 min and washed 8 times. The reactions were developed with TMB substrate (Graphic Scientific, Brisbane, Australia), stopped with IM H₂SO₄, and read at 450 nm using a Microplate EL311autoreader (BioTek, Winooski, VT). Cytokine concentrations were determined against a curve of recombinant standards. For IL-5 the results are expressed as units of a standard recombinant supernatant while the others are in pg. The antibodies used to detect IL-4 were 55434 and 18042D (capture and detection respectively), for IL-5, 18051D and 18062D, for IL-10, 18141D and 18152D, for IL-13, MAB413 and BAF413, and for IFN- γ , 18112D and 18181D. The anti-IL-13 antibodies were obtained from R & D (Minneapolis, MN) and the others were from PharMingen (La Jolla, CA).

Antibody titration

Sera were assayed for IgG1 and IgG2a by ELISA. Maxisorb plates were coated with Der p 1 at 1 μ g/ml in 0.1 M Na₂HPO₄, pH 9.0 and the assay conducted as for the cytokine ELISAs above. The reactivity was developed with biotinylated goat anti-IgG1 (1070-08; Southern Biotechnology Associates, Birmingham, AL) or anti-IgG2a (1080-08; Southern Biotechnology Associates). Dilutions of a pool of hyperimmune sera were used to construct a standard curve and the titers were calculated from the standard which was given an arbitrary concentration of 1000 U/ml.

IgE antibodies were assayed by passive cutaneous anaphylaxis (PCA) or by ELISA. For PCA 0.05 ml of doubling dilutions of sera were injected intradermally into the shaved back of adult male Sprague-Dawley rats. After 24 h the rats

were challenged with 10 mg of spent mite medium in 1 ml of 0.05% Evans Blue (ICN) in saline. The reciprocal of the highest dilution producing a blue spot was taken as the titer and expressed in $\log_2$ units (7). For the ELISA Maxisorb plates (Nunc) were coated with 100 μ l of rat anti-mouse IgE antibody (2 μ g/ml, cat. no. 553413; PharMingen) diluted in 0.1 M Na_2HPO_4 (pH 9.0) and incubated overnight at 4°C. The plates were washed 5 times with 50 mM Tris-HCl, 0.9% NaCl and 0.05% Tween 20, and blocked by adding 250 μ l/well of 2% BSA in 0.05 M Tris-HCl, 0.9% NaCl for 1 h at room temperature. The plates were then washed 5 times, and 100 μ l of either sample or standard diluted in DELFIA Assay buffer (Wallac, Turku, Finland) was added and incubated for 4 h with gentle shaking at room temperature. Dilutions of a pool of hyperimmune sera were used to construct a standard curve and the titers were calculated from the standard which was given a concentration of 100 arbitrary units. The plates were washed 5 times and 100 μ l of Der p 1 (5 μ g/ml) diluted in DELFIA assay buffer added for 2 h at room temperature. After 5 washes, 100 μ l of the biotinylated mouse anti-Der p 1 mAb 4C1 diluted in DELFIA Assay buffer was incubated for 1 h at room temperature. After 5 washes, samples were incubated with 100 μ l of DELFIA europium-labeled streptavidin (Wallac) 30 min with a final 8 washes. Then 200 μ l of DELFIA enhancement solution (Wallac) was added at room temperature. The plates were read 15 min later with a Victor2 1420 Multilabel counter (Wallac) and the titers calculated against the standards.

Delayed hypersensitivity

Mice sensitized with Der p 1 in CFA were challenged on day 7 with an intradermal injection of 10 μ l of Der p 1 in PBS in the ventral side of the pinnae of the ear. After 24 h the thickness of both ears was measured with an engineer's micrometer and the difference taken as a measure of delayed hypersensitivity. The swelling was expressed in units of 10^{-2} mm and compared to the swelling produced by challenging non-sensitized mice.

Bronchoalveolar lavage (BAL)

The lungs of freshly euthanized mice were perfused by injecting PBS through the left atrium of the heart. The airway to the right lobes of the lung was clamped and the right lung was removed and placed in 10% saline-buffered formaldehyde for histological staining. The trachea was exposed and an incision made between the cartilaginous rings immediately below the mandible. A cannula consisting of 2 cm of 1 mm polyethylene tubing (Dural Plastics and Engineering, Dural, NSW, Australia) connected to a 23G needle was inserted, and the left lung was lavaged with 3×300 μ l of PBS/0.2% BSA. For differential cell counts, cytospin preparations of 5×10^4 cells in 100 μ l were made using a cyto centrifuge (Elliot, Shandon, Pittsburg, PA). Cells were dried for 30 min and then stained with Diff Quik (Lab Aids, Narrabeen, Australia) as per the manufacturer's instructions. Paraffin tissue sections were stained with hematoxylin & eosin by standard procedures.

Statistical methods

Groups of five mice were set up for each experiment, which were performed at least in duplicate. The significance was determined by Student's *t*-test or Mann-Whitney tests.

Results

Bystander responses

The intranasal administration of peptides containing the major epitope of Der p 1 has been shown to inhibit the responses of mice sensitized s.c. with Der p 1 in CFA as measured by IL-2 release in *in vitro* recall responses of lymph node cells. The results of previous experiments designed to examine whether or not responses to a bystander antigen injected with the Der p 1 were also inhibited, produced equivocal results with a trend to inhibition at the $P < 0.1$ level (7). To re-examine this, mice were given three intranasal doses of 10 μ g of p114–128 and after 10 days immunized with 100 μ g Der p 1 in CFA containing 100 μ g of OVA. Using this protocol the responses to Der p 1 and OVA could be consistently and significantly inhibited to the same degree as the inhibition of Der p 1 responses (Fig. 1).

Antibody responses to Der p 1 immunization in alum and DTH to immunization in CFA

Mice were given one, three or five daily intranasal doses of 10 μ g of p114–128 or one dose of 50 μ g. At 10 days after the last administration the mice were injected i.p. with 10 μ g of Der p 1 in alum and the ensuing serum antibody responses measured at different time intervals. The IgE titers in mice which received either five intranasal doses or the one 50 μ g dose were 4 $\log_2$ units lower than controls (i.e. an average of 1/16th of controls) and the other groups were the same as the controls (Fig. 2). To confirm the inhibition of IgE, sera were assayed by capture IgE ELISA for anti-Der p 1. There was a close correlation with the PCA. The week 4 titers for the PBS treated group were 725.7 ± 303.9 (mean $\pm$ SD, ELISA units) and for the mice treated with 5×100 μ g peptide 21.58 ± 9.741 units. For comparison, experiments were conducted to determine the effect of different 10 μ g doses of intranasal peptide on delayed hypersensitivity produced after the injection of Der p 1 in CFA. It was also found that five but not three intranasal doses were markedly inhibitory (Table 1). The sera of the same groups of alum immunized mice which were used to show the inhibition of IgE titers were assayed for IgG1 and IgG2a antibody (Figs 3a and 3b). No differences were found between the controls and the intranasal peptide groups.

Respiratory immunization

Respiratory immunization was performed by the intranasal administration of 100 μ g of Der p 1 with 10 μ g of mutated *E. coli* enterotoxin as adjuvant. The titers induced were similar to those induced by the injection of Der p 1 in alum and persisted for at least 8 weeks. Pilot experiments had shown that IgE responses to intranasal Der p 1 alone were transient and variable, and that lower doses of the Der p 1 or the mutated toxin induced lower responses. To examine the effect of intranasal peptides on IgE responses, experiments first were conducted with the same doses and regime of intranasal peptide or Der p 1 administration which had produced the inhibition of IgE and DTH to parenteral immunization. No inhibition was found (data not shown). The dose of intranasal peptide was then increased to five consecutive intranasal doses of 100 μ g. No effect on IgE titer was found (Fig. 4)

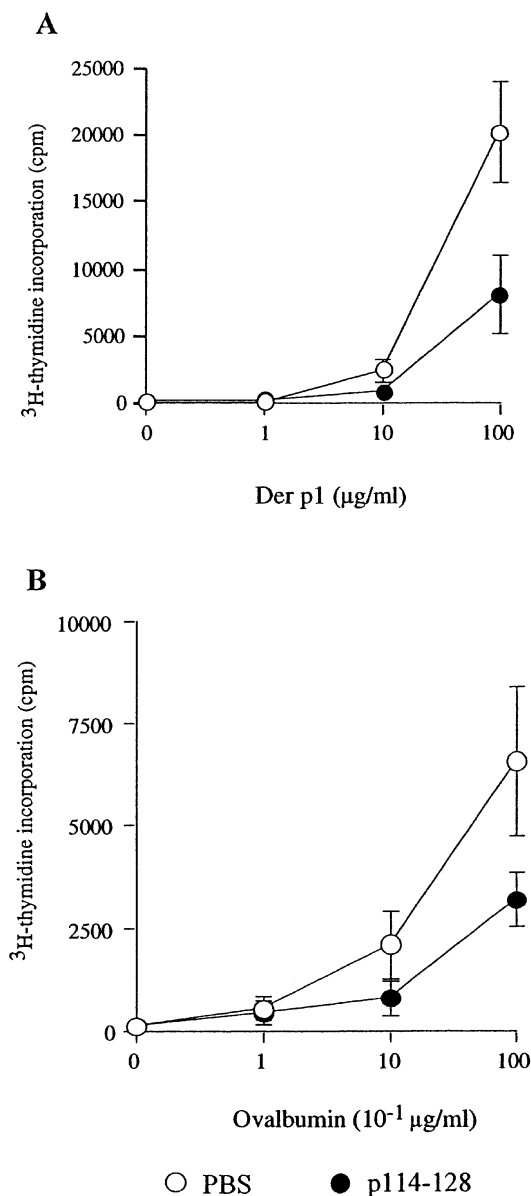


Fig. 1. Bystander suppression. Mice were treated with three daily doses of p114–128 or PBS, and then immunized with a mixture of Der p 1 and OVA in CFA. The results show the mean SD of IL-2 release for groups of five mice. The responses of the peptide-treated group to Der p 1 (A) and OVA (B) were significantly reduced, $P < 0.05$ Student's t -test.

after either a primary or boosted response. The IgG1 antibody titers were not affected by the intranasal peptide but prior intranasal administration of the whole intranasal Der p 1 enhanced the IgG1 titers (Fig. 5a). The IgG2a titers were not affected by intranasal peptide or Der p 1 (Fig. 5b). To test for a possible direct inhibitory effect of the mutated toxin on the induction or maintenance of intranasal tolerance, mice were given five daily doses of intranasal p114–128, and after 10 days treated with intranasal mutated toxin and injected i.p. with Der p 1 in alum. The mutated toxin did not affect the IgE

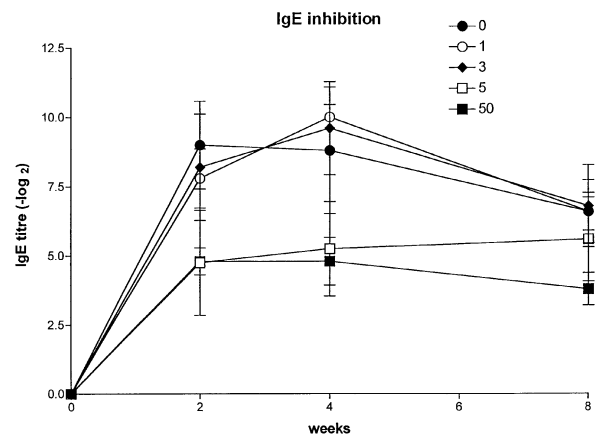


Fig. 2. IgE responses of mice treated with intranasal peptide and immunized with Der p 1 in alum. Mice were treated with one, three or five daily doses of 10 µg of p114–128 (1, 3 or 5) or 1 dose of 50 µg p114–128 (50) or five doses of PBS (0) and injected i.p. with Der p 1 in alum. The IgE titers of groups of five mice (mean $\pm$ SD log₂ titer) were significantly decreased in groups treated with five daily doses of 10 µg peptide or one 50 µg dose, $P < 0.05$ Student's t -test.

Table 1. Effect of different doses of peptide on delayed hypersensitivity

Intranasal treatment	Immunization	Ear swelling (10^{-2} mm)
PBS	Der p 1/CFA	23.7 $\pm$ 2.3
3 $\times$ 10 µg p111–139	Der p 1/CFA	24.1 $\pm$ 1.1
5 $\times$ 10 µg p111–139	Der p 1/CFA	11.5 $\pm$ 1.6
PBS	–	10.7 $\pm$ 1.8
5 $\times$ 10 µg p111–139	–	13.6 $\pm$ 1.9

Groups of five mice were given three or five doses of p111–139 or five doses of PBS and immunized by s.c. injection of 100 µg Der p 1 in CFA. After 7 days they were challenged in the ear with 10 µg of Der p and the ear swelling responses measured after 24 h. The sensitized mice produced significant ear swelling compared to unimmunized controls, $P < 0.05$ Student's t -test, and the five but not three doses of peptide reduced the responses to control levels, $P < 0.05$ Student's t -test.

titers induced by the injected Der p 1 and the inhibition induced by the intranasal peptide was unaffected (Fig. 6).

To examine the effects on recall responses of T cells, mice were given five daily doses of 100 µg of p114–128 and then immunized with 100 µg intranasal Der p 1 and mutated toxin. After 10 days the superficial cervical lymph nodes were removed and responses to Der p 1 were measured by the cytokine released after *in vitro* stimulation. No effect was found on the release of IL-2, IL-5, IL-10 and IFN- γ , but the amount of IL-4 and IL-13 released was reduced to 50% in the group treated with intranasal peptides (Table 2). Replicate experiments produced the same degree of inhibition. To examine lung inflammation, mice were immunized with intranasal Der p 1 with mutant toxin adjuvant and after 4 weeks were challenged with three intranasal doses of 100 µg of Der p 1 given on 3 consecutive days. Two days after the last

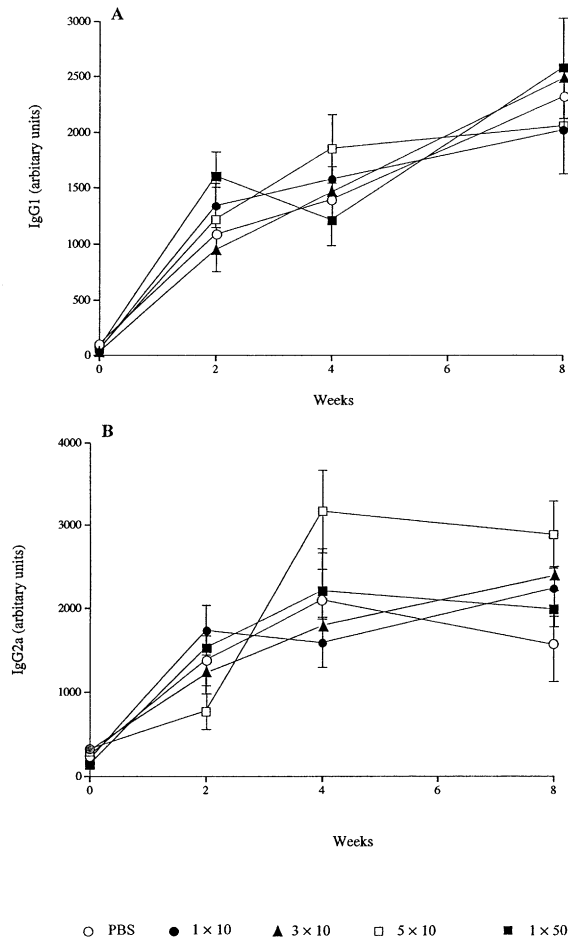


Fig. 3. IgG responses of mice treated with intranasal peptide and immunized with Der p 1 in alum. Mice were treated with one, three or five daily doses of 10 μ g of p114–128 or one dose of 50 μ g p114–128 or five doses of PBS and injected i.p. with 10 μ g Der p 1 in alum. The responses of groups of five mice (mean $\pm$ SD arbitrary ELISA units) show no differences between the groups for IgG1 (A) or IgG2a (B).

instillation the cellular infiltrate was examined by BAL and by histological sections. The infiltrate was primarily lymphocytic. Mice immunized with peptide pretreatment (5 daily doses of 100 μ g) had significantly higher lymphocytic infiltrate ($12,002 \pm 5620$ compared to 988 ± 473 , $P < 0.01$, Mann–Whitney test). The increase was also clearly apparent on examination of histological sections stained with hematoxylin & eosin.

Discussion

The intranasal administration of peptides containing CD4 T cell epitopes can inhibit T_H1 -type immunity as shown by the ability of peptides from myelin basic protein (MBP) to inhibit experimental autoimmune encephalomyelitis (EAE) (1). This peptide pretreatment inhibited both IFN- γ and IL-4 responses and increased IL-10 production, which if blocked by neutralizing antibody prevented the inhibition of disease (11). Liu *et al.* (12) found MBP peptides decreased IFN- γ but without affecting IL-4 and transforming growth factor (TGF)- β produc-

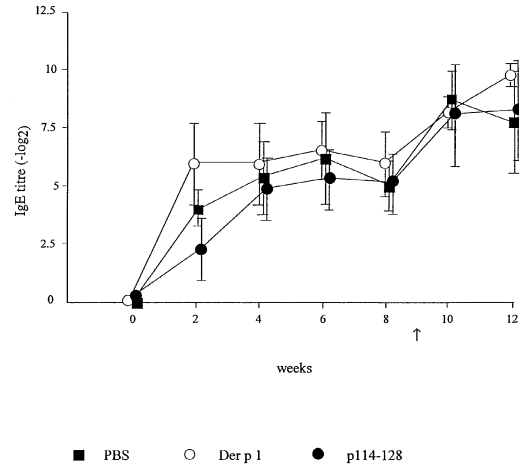


Fig. 4. IgE responses of mice treated with intranasal peptide or antigen and immunized by respiratory sensitization. Mice were given five daily doses of 100 μ g of p114–128 or whole Der p 1 or PBS and then sensitized with the intranasal installation of 100 μ g of Der p 1 with mutated *E. coli* enterotoxin. The IgE titers of all of groups of five mice (mean $\pm$ SD log₂ titer) showed no significant differences in primary or boosted responses (week 9).

tion, and other reports found increased IL-4 and TGF- β responses (2,13). Intranasal peptides from the acetylcholine receptor (AChR) have been shown to prevent the induction of myasthenia gravis (14) and IL-2 release from sensitized T cells, and in a study using whole AChR, intranasal administration decreased IFN- γ and IL-4, while increasing TGF- β (3). In the cases of collagen-induced arthritis (4) and anti-GAD65 responses in spontaneous diabetes (5), intranasal peptides have inhibited disease and converted T_H1 to T_H2 responses by inhibiting IFN- γ and increasing IL-4.

A similar increase in T_H2 responses was indicated by the work of Wolvers with an exogenous antigen, OVA (15). Here intranasal administration of OVA decreased DTH and increased IgE production following immunization with CFA. Previous studies with Der p 1 showed that peptides blocked the induction of DTH in mice immunized with Der p 1 with CFA, but not IgE (7). This type of result and a similar literature on oral tolerance has led to speculation that T_H2 cytokines may be involved in the tolerance or may be more resistant to the toleration. The results here are the first reports of the effect of intranasal synthetic peptide on a T_H2 immunization protocol and show IgE can be readily inhibited with a low-dose regimen or by a single high-dose application, by at least 80%.

The initial studies on Der p 1 peptides examined responses to the allergen injected in CFA because this regimen readily induced responses to individual T cell epitopes which could be measured with different peptides. It was shown that peptides containing only one T cell epitope could markedly inhibit sensitization to all epitopes on Der p 1 as measured by *in vitro* IL-2 release or by *in vivo* DTH (6,7). This intramolecular suppression, where regulatory responses to one epitope blocked responses to the whole molecule, as well as the ability of the peptides to block responses of primed mice (6), has suggested some utility in modifying hypersensitivity

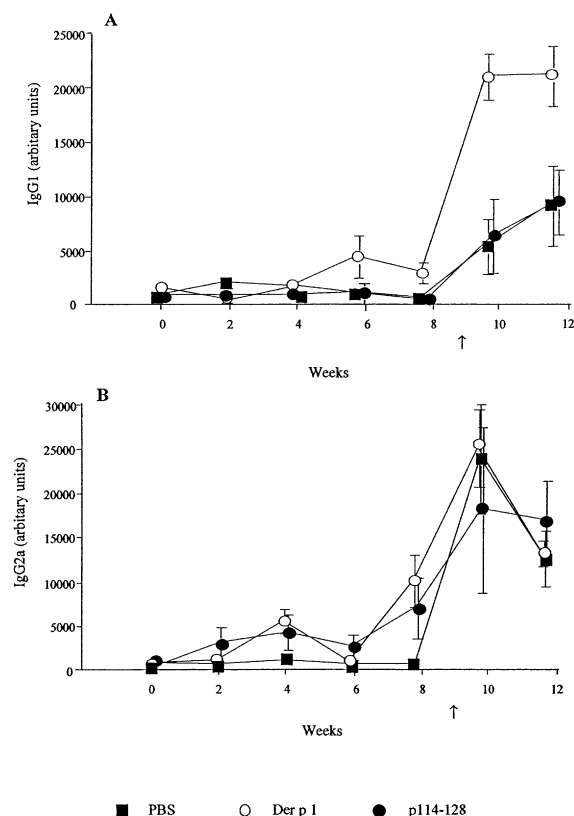


Fig. 5. IgG responses of mice treated with intranasal peptide or antigen and immunized by respiratory sensitization. Mice were given five daily doses of 100 μ g of p114–128, whole Der p 1 or PBS and then sensitized with the intranasal installation of 100 μ g of Der p 1 with mutated *E. coli* enterotoxin. The responses of groups of five mice (mean $\pm$ SD arbitrary ELISA units) show that (A) intranasal pretreatment with Der p 1 enhanced the IgG1 responses when boosted (week 9) while peptide had no effect and (B) no differences were found for IgG2a responses.

reactions to complex antigens. It is, however, important to study IgE responses to a T_H2 -type immunization. The results here show that the daily installation of five quite low doses (10 μ g) of peptide can inhibit IgE titers, induced by the i.p. injection of Der p 1 in alum. The intranasal doses required were the same as those required for the inhibition of DTH in animals sensitized with allergen in CFA. A possible reason why many studies have shown that T_H2 cytokine responses can be increased in responses induced to antigen in CFA is that there is that is a release from regulation by the dominant T_H1 responses. It is also possible that mechanisms of intranasal and oral tolerance require the injection of antigen to become activated, and this, depending on whether it was a T_H1 or T_H2 stimulus, could influence the inhibitory activity.

Although this is the first report of the effect of the intranasal administration of synthetic peptides on IgE responses, the effect of intranasal antigen or aerosols has been previously studied. Holt *et al.* (16) originally found that chronic intranasal administration of OVA inhibited IgE responses of mice injected with antigen in alum and that chronic intranasal Der p 1 could inhibit IgE responses of low responder rats (17), although

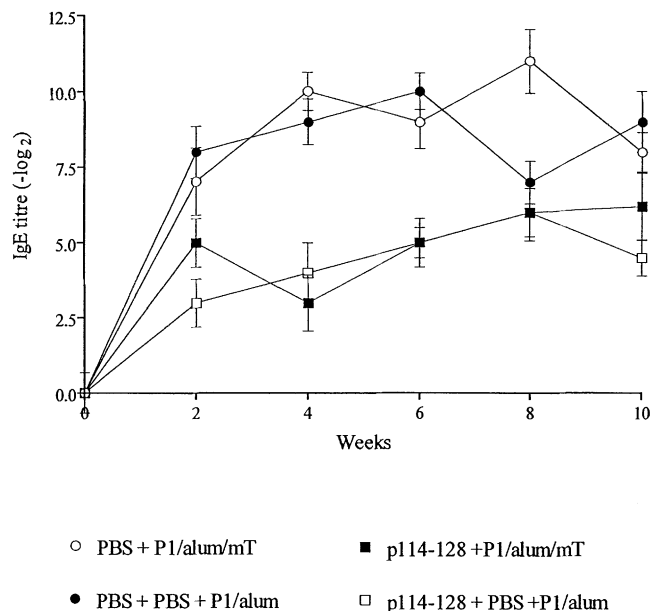


Fig. 6. Effect of mutant toxin adjuvant on the peptide-induced inhibition of parenteral IgE responses. Mice were given five daily intranasal doses of 100 μ g of p114–128 or PBS and then injected i.p. with 10 μ g Der p 1 in alum. At the time of the i.p. injection groups were given either intranasal PBS (+PBS) or the mutant enterotoxin (mT). The IgE titers of all of groups of five mice (mean $\pm$ SD log₂ titer) showed that IgE was inhibited by the peptide treatment $P < 0.05$ Student's *t*-test. The treatment with mutated enterotoxin did not affect the IgE titers in the peptide or PBS pretreated groups.

responses of high responders and mice were unaffected. Like the results described here for high-responder mice, IgG was not inhibited. Although some contribution from ingested allergen could not be excluded, as is the case here, the dose of allergen ingested was shown to be far lower than that required in standard oral tolerance regimens (16). Later studies showed that OVA aerosols produced a similar effect which was associated with the development of IFN- γ -producing cells (18). Recent studies using a short intranasal exposure protocol similar to that used here have also found selective IgE tolerance antibody (19,20) but IFN- γ was not increased (20). In contrast, the study of Wiedermann (21) with whole intranasal Bet v 1 allergen found that IgE and all IgG isotypes were markedly reduced, as well as T cell proliferation and the release of IL-4, IL-5, IL-10 and IFN- γ in *in vitro* stimulation assays. The ability to inhibit responses with intranasal peptides differs very significantly from the reported effect of s.c. injection of peptide on T_H2 responses to OVA. The injection of peptide containing the dominant OVA epitope stimulated instead of inhibited IgE responses (22). Inhibition could only be achieved by injecting the whole protein. In the experiments here, the intranasal administration of the whole protein, but not peptide, significantly stimulated IgG1 produced by respiratory immunization. The use of peptide may therefore be preferable for aeroallergen immunotherapy.

The demonstration of bystander inhibition here confirms previous experiments which showed a strong trend to inhibition (7). The responses of both the bystander protein and Der p 1 were similarly inhibited. The mechanism may be the

Table 2. Effect of peptide treatment on cytokine production in mice sensitized by the respiratory mucosa

Intranasal pretreatment	IL-2 (c.p.m.)	IL-4 (pg/ml)	IL-5 (U/ml)	IL-10 (pg/ml)	IL-13 (pg/ml)	IFN- γ (pg/ml)
PBS	12,521 $\pm$ 1454	8.2 $\pm$ 0.9	824 $\pm$ 140.3	291 $\pm$ 54.5	235.6 $\pm$ 13.6	804.6 $\pm$ 205.0
5 $\times$ p114-128	9765 $\pm$ 1381	4.3 $\pm$ 1.3	896.1 $\pm$ 75.4	307 $\pm$ 76.0	116.7 $\pm$ 20.8	1089.8 $\pm$ 308.7

Groups of five mice were given five daily administrations of 100 μ g of p114-128 or PBS and then sensitized by intranasal administration of 100 μ g of Der p 1 with mutated enterotoxin adjuvant. After 10 days the cervical lymph nodes were removed and assayed for cytokine release in *in vitro* assays stimulated with Der p 1. The titers (mean $\pm$ SD) are [^{3} H]thymidine incorporation for IL-2, arbitrary ELISA units for IL-5 titrated against a recombinant IL-5 supernatant and pg/ml for the other cytokines calculated from known standards. The IL-4 and IL-13 titers were significantly lower in the peptide-treated groups, $P < 0.05$ Students *t*-test.

same or different to the intramolecular suppression described previously. One mechanism is that the inhibition can be mediated by contact inhibition signals mediated by the Notch pathway at the time of antigen presentation (23). Clearly this could also exist if the co-administered antigens were processed and presented by the same antigen-presenting cells. The production of immunosuppressive TGF- β has not been detected in the Der p 1 model, but regulation by IL-10 as described by Burkhart *et al.* (11) in the EAE model could be another mechanism.

All previous studies on intranasal inhibition have only examined responses induced by parenteral sensitization. Although the experiments of Wiedermann *et al.* (21) with whole Bet v 1 allergen used a respiratory challenge, the response was critically dependent on i.p. sensitization in alum. Immunoregulation of responses induced via the respiratory mucosa can be different to other sites. Wolvers *et al.* (24) have directly shown by lymph node transplantation that, for producing intranasal tolerance, the respiratory nodes cannot be substituted by nodes draining other tissues. Constant *et al.* (25) have reported that C57BL/6J mice develop T_H2 responses to the respiratory inoculation of *Leishmania* instead of the strong protective T_H1 responses induced by injection. The circulation of cells may also be different. The nodes draining the upper respiratory tract (15,26) do produce some MadCAM integrin, the ligand for $\alpha_4\beta_7$ integrin, found in the gut, and T cells in the epithelium have the $\alpha_E\beta_7$ integrin also found in the gut (27). Most T cells in lung inflammation have the $\alpha_4\beta_1$ integrin which is found on memory cells which circulate to most tissues (28,29) and the $\alpha_E\beta_7$ does not therefore appear to be of paramount importance (30,31). T cells with the $\alpha_E\beta_7$ -integrin, however, do predominate in the bronchial epithelium along with the epithelial ligand E-selectin (27). While the function of these cells has not been ascertained they can persist in the epithelium where they can be regulated by TGF- β (32). The gut intraepithelial T cells have been shown to respond to down-regulatory signals from the epithelium which do not affect spleen T cells so it is possible the $\alpha_E\beta_7$ -bearing intraepithelial T cells may have a role in dampening mucosal responses (33). Other studies have indicated that while the homing of memory cells, such as those with $\alpha_4\beta_1$ integrin, may not be tissue specific there is a mechanism for the preferential survival of cells in the tissue of priming (46). In a novel mechanism found for stromal-derived factor-1, low concentrations of chemokines can attract while high concentrations can repulse the migration of T cells (34). This has not been studied in the lung, but the eosinophil influx into lung

inflammation can be blocked by anti-CXC4R, the only known ligand for SDF-1 (35). Another factor to consider is that cytokine delivered to or produced in the mucosa can prevent the induction of mucosal tolerance and lead to immune responses (20,36).

Previous experiments had shown that the intranasal administration of Der p 1 led to transient but substantial immune responses in the draining lymph nodes and spleen. The T cells from these tissues could release IL-2, IFN- γ and granulocyte macrophage colony stimulating factor (GM-CSF), but no IL-4 or TGF- β was detected. A further intranasal exposure to peptide after 21 days induced a similar immune response where, in contrast, parenteral challenge at this time with Der p 1 or peptide in CFA showed the characteristic reduction in responses. The current experiments were designed to examine the effect of the peptides on an immunogenic mucosal stimulation capable of inducing persistent responses, similar to injection in adjuvant. The responses induced by a single intranasal administration of Der p 1 with mutated enterotoxin elicited IgE titers similar to those induced by the injection of Der p 1 in alum. The administration of peptide did not alter the IgE titer in response to this sensitization or the production of IgG1 and IgG2a antibody isotypes. Thus, while 5 $\times$ 10 μ g or 1 $\times$ 50 μ g could inhibit the IgE production to immunization with Der p 1 in alum or DTH to Der p 1 in CFA, neither these doses or a very high dose regimen of 5 $\times$ 100 μ g of peptide affected antibody production. The administration of the mutated enterotoxin adjuvant itself did not abrogate the mucosal regulation because administration of the mutated toxin when the immunization was by injection with alum did not affect the inhibition.

While the intranasal peptide did not inhibit the IgE responses to respiratory immunization it did have some effect on T_H2 cytokines. When the cells from the draining lymph nodes were tested for their ability to produce cytokines *in vitro* there was no difference in the release of IL-2, IL-5, IL-10 or IFN- γ , but there was a consistent reduction in their ability to release IL-4 and IL-13, to about half the level of control responses. This clearly was not sufficient to reduce IgE antibody but IL-4 and IL-13 have many direct effects on mucosal inflammation such as the induction of chemokines by epithelial cells including eotaxin (37), the induction of adhesion molecules VCAM-1 (38,39) and ICAM-1 (40) on epithelial and vascular cells, the induction of 15-lipoxygenase and eicosanoid inflammatory mediators (41), the stimulation of mucus secretion (42,43), the induction of GM-CSF (39) and effects on tissue matrix (44). Indeed, aerosols of soluble IL-4R has been

shown to produce rapid improvements in asthmatic lung function indicating an effect beyond the regulation of lymphocyte differentiation (45). The knowledge that these cytokine responses can be decreased by intranasal peptides also indicates that it is possible that a more efficient protocol may be devised. The peptides unexpectedly did not inhibit IL-2 and IFN- γ and thus a more chronic treatment may skew responses away from the T_H2 -type 1 hypersensitivity. The lungs of mice pretreated with peptide also had an increased lymphocyte infiltrate after challenge with Der p 1. This is a potentially important result showing the need to examine appropriate models of sensitization. More detailed studies on the activity of these cells and their effect on respiratory function are, however, required. The split cytokine regulation has been previously evident in that despite the marked inhibition of IL-2 in responses to immunization with CFA, the intranasal peptide administration has consistently had little effect on GM-CSF/IL-3 release (6). The lack of any increase in IL-10 production is of interest because of the contrast with the marked increase found in mice treated with intranasal peptide and immunized with MPB in CFA (11). The regulatory effects manifested by intranasal peptide appear to depend on the type of sensitization.

In summary, therefore, the intranasal administration of peptide containing the major T cell epitope of the allergen Der p 1 can readily inhibit IgE production to a parenteral injection of Der p 1 in alum as well the inhibition of DTH responses induced by the injection of antigen in CFA. The peptide administration could inhibit some responses induced by sensitization via the respiratory mucosa, as shown for IL-4 and IL-13 but even high doses of intranasal peptide did not affect IgE via this route. Although the route and method of antigen challenge has important quantitative and qualitative effects on the outcome, immunomodulation was observed for both type 1 and cell-mediated hypersensitivity responses.

Acknowledgement

This work was supported by the National Health and Medical Research Council of Australia.

Abbreviations

AChR	acetylcholine receptor
BAL	bronchoalveolar lavage
CFA	complete Freund's adjuvant
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
GM-CSF	granulocyte macrophage colony stimulating factor
MBP	myelin basic protein
OVA	ovalbumin
PCA	passive cutaneous anaphylaxis
TGF	transforming growth factor

References

- Metzler, B. and Wraith, D. C. 1993. Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Int. Immunol.* 5:1159.
- Li, H. L., Liu, J. Q., Bai, X. F., van der Meide, P. H. and Link, H. 1998. Dose-dependent mechanisms relate to nasal tolerance induction and protection against experimental autoimmune encephalomyelitis in Lewis rats. *Immunology* 94:431.
- Ma, C. G., Zhang, G. X., Xiao, B. G. and Link, H. 1996. Cellular mRNA expression of interferon-gamma (IFN-gamma), IL-4 and transforming growth factor-beta (TGF-beta) in rats nasally tolerized against experimental autoimmune myasthenia gravis (EAMG). *Clin. Exp. Immunol.* 104:509.
- Myers, L. K., Seyer, J. M., Stuart, J. M. and Kang, A. H. 1997. Suppression of murine collagen-induced arthritis by nasal administration of collagen. *Immunology* 90:161.
- Tian, J., Atkinson, M. A., Clare-Salzler, M., Herschenfeld, A., Forsthuber, T., Lehmann, P. V. and Kaufman, D. L. 1996. Nasal administration of glutamate decarboxylase (GAD65) peptides induces T_H2 responses and prevents murine insulin-dependent diabetes. *J. Exp. Med.* 183:1561.
- Hoyne, G. F., O'Hehir, R. E., Wraith, D. C., Thomas, W. R. and Lamb, J. R. 1993. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J. Exp. Med.* 178:1783.
- Hoyne, G. F., Jarnicki, A. G., Thomas, W. R. and Lamb, J. R. 1997. Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. *Int. Immunol.* 9:1165.
- Hoyne, G. F., Askonas, B. A., Hetzel, C., Thomas, W. R. and Lamb, J. R. 1996. Regulation of house dust mite responses by intranasally administered peptide: transient activation of CD4⁺ T cells precedes the development of tolerance *in vivo*. *Int. Immunol.* 8:335.
- Hoyne, G. F. and Thomas, W. R. 1995. T cell responses to orally administered antigens. Study of the kinetics of lymphokine production after single and multiple feeding. *Immunology* 84:304.
- Tsuji, T., Yokochi, T., Kamiya, H., Kawamoto, Y., Miyama, A. and Asano, Y. 1997. Relationship between a low toxicity of the mutant A subunit of enterotoxigenic *Escherichia coli* enterotoxin and its strong adjuvant action. *Immunology* 90:176.
- Burkhart, C., Liu, G. Y., Anderton, S. M., Metzler, B. and Wraith, D. C. 1999. Peptide-induced T cell regulation of experimental autoimmune encephalomyelitis: a role for IL-10. *Int. Immunol.* 11:1625.
- Liu, J. Q., Bai, X. F., Shi, F. D., Xiao, B. G., Li, H. L., Levi, M., Mustafa, M., Wahren, B. and Link, H. 1998. Inhibition of experimental autoimmune encephalomyelitis in Lewis rats by nasal administration of encephalitogenic MBP peptides: synergistic effects of MBP 68-86 and 87-99. *Int. Immunol.* 10:1139.
- Bai, X. F., Shi, F. D., Xiao, B. G., Li, H. L., van der Meide, P. H. and Link, H. 1997. Nasal administration of myelin basic protein prevents relapsing experimental autoimmune encephalomyelitis in DA rats by activating regulatory cells expressing IL-4 and TGF-beta mRNA. *J. Neuroimmunol.* 80:65.
- Barchan, D., Souroujon, M. C., Im, S. H., Antozzi, C. and Fuchs, S. 1999. Antigen-specific modulation of experimental myasthenia gravis: nasal tolerization with recombinant fragments of the human acetylcholine receptor alpha-subunit. *Proc. Natl Acad. Sci. USA* 96:8086.
- Wolvers, D. A., van der Cammen, M. J. and Kraal, G. 1997. Mucosal tolerance is associated with, but independent of, up-regulation T_H2 responses. *Immunology* 92:328.
- Holt, P. G., Batty, J. E. and Turner, K. J. 1981. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 42:409.
- Stewart, G. A. and Holt, P. G. 1987. Immunogenicity and tolerogenicity of a major house dust mite allergen, Der p I from *Dermatophagoides pteronyssinus*, in mice and rats. *Int. Arch. Allergy Appl. Immunol.* 83:44.
- McMenamin, C. and Holt, P. G. 1993. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8⁺ T cell-mediated but MHC class II-restricted CD4⁺ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J. Exp. Med.* 178:889.
- van Halteren, A. G., van der Cammen, M. J., Cooper, D., Savelkoul, H. F., Kraal, G. and Holt, P. G. 1997. Regulation of antigen-

- specific IgE, IgG1, and mast cell responses to ingested allergen by mucosal tolerance induction. *J. Immunol.* 159:3009.
- 20 Tsitoura, D. C., DeKruyff, R. H., Lamb, J. R. and Umetsu, D. T. 1999. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled $CD4^+$ T cells. *J. Immunol.* 163:2592.
 - 21 Wiedermann, U., Jahn-Schmid, B., Bohle, B., Repa, A., Renz, H., Kraft, D. and Ebner, C. 1999. Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant Bet v 1, the major birch pollen allergen, in a murine model of type I allergy. *J. Allergy Clin. Immunol.* 103:1202.
 - 22 Janssen, E. M., Wauben, M. H., Jonker, E. H., Hofman, G., Van Eden, W., Nijkamp, F. P. and Van Oosterhout, A. J. 1999. Opposite effects of immunotherapy with ovalbumin and the immunodominant T cell epitope on airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. *Am. J. Respir. Cell. Mol. Biol.* 21:21.
 - 23 Forsyth, L. M., Dallman, M. J., Owen, M. J., Ish-Horowicz, D. and Lamb, J. R. 2000. Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral $CD4^+$ T cells. *Int. Immunol.* 12:177.
 - 24 Wolvers, D. A., Coenen-de Roo, C. J., Mebius, R. E., van der Cammen, M. J., Tirion, F., Miltenburg, A. M. and Kraal, G. 1999. Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39. *J. Immunol.* 162:1994.
 - 25 Constant, S. L., Lee, K. S. and Bottomly, K. 2000. Site of antigen delivery can influence T cell priming: pulmonary environment promotes preferential T_H2 -type differentiation. *Eur. J. Immunol.* 30:840.
 - 26 Csencsits, K. L., Jutila, M. A. and Pascual, D. W. 1999. Nasal-associated lymphoid tissue: phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to high endothelial venules in a mucosal site. *J. Immunol.* 163:1382.
 - 27 Erle, D. J. and Pabst, R. 2000. Intraepithelial lymphocytes in the lung: a neglected lymphocyte population [Comment]. *Am. J. Respir. Cell. Mol. Biol.* 22:398.
 - 28 Feng, C. G., Britton, W. J., Palendira, U., Groat, N. L., Briscoe, H. and Bean, A. G. 2000. Up-regulation of VCAM-1 and differential expansion of beta integrin-expressing T lymphocytes are associated with immunity to pulmonary *Mycobacterium tuberculosis* infection. *J. Immunol.* 164:4853.
 - 29 Nakajima, H., Sano, H., Nishimura, T., Yoshida, S. and Iwamoto, I. 1994. Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue. *J. Exp. Med.* 179:1145.
 - 30 Wagner, N., Lohler, J., Kunkel, E. J., Ley, K., Leung, E., Krissansen, G., Rajewsky, K. and Muller, W. 1996. Critical role for beta7 integrins in formation of the gut-associated lymphoid tissue. *Nature* 382:366.
 - 31 Abitorabi, M. A., Mackay, C. R., Jerome, E. H., Osorio, O., Butcher, E. C. and Erle, D. J. 1996. Differential expression of homing molecules on recirculating lymphocytes from sheep gut, peripheral, and lung lymph. *J. Immunol.* 156:3111.
 - 32 Goto, E., Kohrogi, H., Hirata, N., Tsumori, K., Hirosako, S., Hamamoto, J., Fujii, K., Kawano, O. and Ando, M. 2000. Human bronchial intraepithelial T lymphocytes as a distinct T cell subset: their long-term survival in SCID-Hu chimeras [see Comments]. *Am. J. Respir. Cell. Mol. Biol.* 22:405.
 - 33 Yamamoto, M., Fujihashi, K., Kawabata, K., McGhee, J. R. and Kiyono, H. 1998. A mucosal intranet: intestinal epithelial cells down-regulate intraepithelial, but not peripheral, T lymphocytes. *J. Immunol.* 160:2188.
 - 34 Poznansky, M. C., Olszak, I. T., Foxall, R., Evans, R. H., Luster, A. D. and Scadden, D. T. 2000. Active movement of T cells away from a chemokine. *Nat. Med.* 6:543.
 - 35 Gonzalo, J. A., Lloyd, C. M., Peled, A., Delaney, T., Coyle, A. J. and Gutierrez-Ramos, J. C. 2000. Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease. *J. Immunol.* 165:499.
 - 36 Stampfli, M. R., Wiley, R. E., Neigh, G. S., Gajewska, B. U., Lei, X. F., Snider, D. P., Xing, Z. and Jordana, M. 1998. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest.* 102:1704.
 - 37 Li, L., Xia, Y., Nguyen, A., Lai, Y. H., Feng, L., Mosmann, T. R. and Lo, D. 1999. Effects of T_H2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J. Immunol.* 162:2477.
 - 38 Barks, J. L., McQuillan, J. J. and Iademarco, M. F. 1997. TNF-alpha and IL-4 synergistically increase vascular cell adhesion molecule-1 expression in cultured vascular smooth muscle cells. *J. Immunol.* 159:4532.
 - 39 Doucet, C., Brouty-Boye, D., Pottin-Clemenceau, C., Jasmin, C., Canonica, G. W. and Azzarone, B. 1998. IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int. Immunol.* 10:1421.
 - 40 Striz, I., Mio, T., Adachi, Y., Heires, P., Robbins, R. A., Spurzem, J. R., Illig, M. J., Romberger, D. J. and Rennard, S. I. 1999. IL-4 induces ICAM-1 expression in human bronchial epithelial cells and potentiates TNF-alpha. *Am. J. Physiol.* 277:L58.
 - 41 Profita, M., Vignola, A. M., Sala, A., Mirabella, A., Siena, L., Pace, E., Folco, G. and Bonsignore, G. 1999. Interleukin-4 enhances 15-lipoxygenase activity and incorporation of 15(S)-HETE into cellular phospholipids in cultured pulmonary epithelial cells. *Am. J. Respir. Cell. Mol. Biol.* 20:61.
 - 42 Temann, U. A., Prasad, B., Gallup, M. W., Basbaum, C., Ho, S. B., Flavell, R. A. and Rankin, J. A. 1997. A novel role for murine IL-4 *in vivo*: induction of MUC5AC gene expression and mucin hypersecretion. *Am. J. Respir. Cell. Mol. Biol.* 16:471.
 - 43 Zhu, Z., Homer, R. J., Wang, Z., Chen, Q., Geba, G. P., Wang, J., Zhang, Y. and Elias, J. A. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* 103:779.
 - 44 Ong, C., Wong, C., Roberts, C. R., Teh, H. S. and Jirik, F. R. 1998. Anti-IL-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma. *Eur. J. Immunol.* 28:2619.
 - 45 Borish, L. C., Nelson, H. S., Lanz, M. J., Claussen, L., Whitmore, J. B., Agosti, J. M. and Garrison, L. 1999. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. *Am. J. Respir. Cell. Mol. Biol.* 160:1816.
 - 46 Bode, U., Wonigeit, K., Pabst, R. and Westermann, J. 1997. The fate of activated T cells migrating through the body: rescue from apoptosis in the tissue of origin. *Eur. J. Immunol.* 27:2087.