Brief Definitive Report

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

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Summary

Antigen-specific $CD4^+$ T cells play an important role in the allergic immune response to house dust mite (HDM) allergens in humans. The group 1 allergen of *Dermatophagoides* spp. is a major target antigen in both B and T cell recognition of HDM. In vitro studies have shown that the presentation of peptides to human T cells under appropriate conditions may lead to a state of specific nonresponsiveness. Therefore, to determine if peptides are able to modulate the function of allergen-reactive T cells in vivo, we have used a murine model of T cell recognition of the HDM allergen Der p 1. The results demonstrate that inhalation of low concentrations of peptide containing the major T cell epitope of Der p 1 (residues 111–139), induces tolerance in naive C57BL/6J mice such that they become profoundly unresponsive to an immunogenic challenge with the intact allergen. When restimulated in vitro with antigen, lymph node T cells isolated from tolerant mice secrete very low levels of interleukin 2, proliferate poorly, and are unable to provide cognate help to stimulate specific antibody production. Furthermore, intranasal peptide therapy was able to inhibit an ongoing immune response to the allergen in mice and this has potential implications in the development of allergen-based immunotherapy.

Approximately 10% of the population suffers from allergy to house dust mite (HDM) and $CD4^+$ T cells are known to play an important role in allergic sensitization (1). In vitro studies have shown that human HDM-reactive $CD4^+$ T cell clones can be rendered unresponsive by exposure to either high doses of their specific peptide epitope or to superantigens (2–6). Therefore, by modulating the function of allergen-reactive T cells in vivo it may be possible to control an established allergic immune response in humans. Independent studies have shown that immunogenic peptides can preferentially induce antigen-specific T cell tolerance in naive mice when administered either intraperitoneally in IFA or intravenously (7–9). Experimental animals may also be rendered immunologically tolerant by feeding or inhaling antigens, and this state of nonresponsiveness is usually long-lasting and antigen specific (10–13). Experimental allergic encephalomyelitis (EAE) is a demyelinating autoimmune disease of rodents, and $CD4^+$ T cells specific for myelin basic protein (MBP) are responsible for the development of disease. Rats can be protected from developing EAE by oral administration of MBP before an encephalitogenic challenge from the autoantigen (14, 15). Similarly, susceptible $H2^u$ mice can be protected from developing EAE by inhalation but not oral administration of a peptide containing the immunodominant T cell epitope on MBP (Ac 1–9, 1–11) (16). In a previous study, we have shown, using a murine model, that T cell responses to the Der p 1 allergen were inhibited by feeding recombinant peptides containing either major or minor T cell epitopes (Hoyne, G. F., M. G. Callow, M.-C. Kuo, and W. R. Thomas, manuscript submitted for publication). The induction of tolerance resulted in decreased responses in LN T cells to the whole antigen in vitro, and a loss of reactivity to all T cell epitopes on the antigen was observed.

In this study, we show that if naive C57BL/6J mice inhale small quantities of synthetic peptides containing T cell epitopes, they become profoundly unresponsive to a powerful immunogenic challenge with the allergen. LN T cells from tolerized mice secrete low levels of IL-2 and proliferate poorly when restimulated with antigen. They are also unable to provide help for antibody production in vitro. Furthermore, in-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/11/1783/06 $2.00
Volume 178 November 1993 1783–1788

tranasal peptide therapy inhibited T cell responses in mice that had been previously sensitized to the Der p 1 allergen. These studies indicate that administering immunogenic peptides intranasally may be an effective way to control aberrant immune responses that give rise to specific disease states in humans.

Materials and Methods

Animals. Inbred C57BL/6J mice were purchased from the Animal Resource Centre (Murdoch, Western Australia) at 6–8 wk of age and were kept under specific pathogen-free conditions.

Antigens. The house dust mite allergen Der p 1 was affinity purified from spent mite medium (17). Synthetic peptides derived from the Der p 1 sequence were synthesized using standard F-moc chemistry, peptides were purified by reverse-phase HPLC, and the sequence of individual peptides were confirmed by sequencing. The peptides used in this study were p1 111–139, p1 156–168 of the group 1 allergen of *Dermatophagoides pteronyssinus* (Der p 1) and p2 21–35 from the group 2 allergen (Der p 2).

Preparation of Recombinant Proteins. A cDNA insert encoding the 57–130 fragment of the Der p 1 protein was ligated to the pGEX vector which expresses the fusion proteins with glutathione-S-transferase in *Escherichia coli*. (18). The procedures for the cloning and expression of the fragments have been described (19). Log phase *E. coli* cells transformed with the pGEX-based construct were induced to express the recombinant protein by adding 0.1 mM isopropylthiogalactosidase (Promega Corp., Madison, WI). The fusion protein was purified to homogeneity using glutathione-coupled agarose (Sigma Chemical Co., St. Louis, MO) following the techniques previously described by Smith and Johnson (18), and eventually lyophilized.

Induction of Intranasal Tolerance. Mice were lightly anesthesized under ether and peptides dissolved in PBS were administered intranasally in a total volume of 10 μl using a micropipette on three consecutive days. Mice were immunized subcutaneously at the base of tail 7 d after the last treatment with 100 μg of Der p 1 emulsified in CFA (Difco, Detroit, MI) in a volume of 0.2 ml. To inhibit ongoing responses, mice were immunized intraperitoneally with Der p 1 in CFA, and 10 d later received 100 μg of p1 111–139 intranasally on five consecutive days. Mice were then reimmunized 7 d later with 100 μg of Der p 1 in IFA (Difco) subcutaneously at the base of tail.

Culture Medium. LN cells were cultured in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 2% FCS (Commonwealth Serum Laboratories [CSL], Parkville, Victoria, Australia), 50 μM 2-ME (Sigma Chemical Co.), 2 mM L-glutamine (CSL), and 20 μg/ml gentamycin (David Bull Laboratories, Mulgrave, Victoria, Australia). CTLL-2 cells were maintained in RPMI (GIBCO BRL) plus 10% FCS.

T Cell Assays. The periaortic and inguinal LNs were expressed through a stainless steel wire mesh, washed, and cultured at 4×10^5 cells in a volume of 0.2 ml in culture medium in a 96-well flat-bottom tissue culture plate (Becton Dickinson Labware, Lincoln Park, NJ). Protein or peptide antigens were added at various concentrations and the cells were incubated at 37°C for 24 h. Supernatants were collected and stored at −20°C until required for the assay.

Lymphokine Assays. The CTLL-2 cell line proliferates maximally with IL-2 but only poorly in the presence of IL-4 (20). 5×10^3 CTLL-2 cells per well were cultured in supernatants for 24 h at 37°C and pulsed with 1 μCi of [^{3}H]thymidine. Cells were harvested onto glass fiber filters and the proliferation determined by incorporation of [^{3}H]thymidine which was assessed using a direct beta counter (Packard Matrix 9600; Packard Instruments, Meriden, CT).

In Vitro Antibody Production. Mice were immunized with 100 μg of Der p 1 in CFA and 10 d later, the spleens of two mice were pooled and RBCs were lysed with sterile distilled water. Periaortic and inguinal LN cells were isolated 7 d after immunization from mice that had been treated on three consecutive days with either PBS (control) or p1 111–139 (tolerant) intranasally and immunized subcutaneously 7 d later with Der p 1/CFA. 2×10^6 spleen or LN cells were cultured alone in the presence of Der p 1 in a volume of 1 ml culture medium in a 24-well tissue culture plate. Alternatively, 10^6 spleen cells were mixed with 10^6 LN cells from control or tolerant mice and cultured in a 1-ml volume with the addition of antigen. Cells were cultured for 7 d at 37°C and the supernatants were assayed for the presence of Der p 1-specific Abs using an ELISA.

ELISA. Affinity-purified protein was diluted in 50 mM Na HCO_3 buffer, pH 9.6, and coated onto an ELISA plate at 100 μg/ml in a volume of 50 μl/well. The plate was incubated overnight at 4°C and the next day was washed three times with PBS plus 0.05% Tween 20. Nonspecific binding sites were blocked by incubating the plate with a solution of PBS containing 1% BSA for 1 h at room temperature. After three washes, serum was added to the plate at appropriate dilutions and incubated for 2 h at room temperature. The plate was washed as described and a 1/1,000 dilution of goat anti-mouse Ig conjugated to horseradish peroxidase was added and incubated for 1 h at room temperature. After a final wash, the color was developed with ABTS containing 1% hydrogen peroxide for 20 min at room temperature. The reaction was stopped by addition of 0.1 M citric acid plus 0.01% sodium azide, and the OD of the plate was measured at 405 nm using an ELISA plate reader. Results are expressed as ELISA OD units.

Results and Discussion

C57BL/6J mice are high responders to the HDM allergen Der p 1 and T cells recognize three different epitopes that are located within the following sequences: 110–131, 78–100, and 21–49 (Hoyne, G. F., et al., manuscript submitted for publication). The ability of purified recombinant GEX p57–130 and a synthetic peptide that contains the immunodominant determinant, p1 111–139 to induce tolerance when administered intranasally were compared. This epitope was chosen for study because it represents a major T cell epitope recognized by human Der p 1-specific T cell clones (6). Mice received 100 μg of either peptide or PBS intranasally on three consecutive days and 1 wk later, all mice were challenged with Der p 1 in CFA. LN cells draining the site of injection were collected 7 d later and stimulated in vitro with either protein or peptide for 24 h and the supernatants were assayed for IL-2. LN cells from mice treated with either p1 111–139 or GEX p57–130 failed to respond with either Der p 1 (Fig. 1 *A*) or the T cell epitopes p1 111–139, p1 78–100 and p1 21–49 (Fig. 1, *B–D*). Control mice that received PBS intranasally developed strong IL-2 responses to both the intact protein and the peptides (Fig. 1).

In addition, mice received either PBS or p1 111–139 intranasally and 1 wk later were challenged subcutaneously with an immunogenic concentration of p1 111–139. LN cells were cultured in vitro with p1 111–139 and IL-2 secretion and T

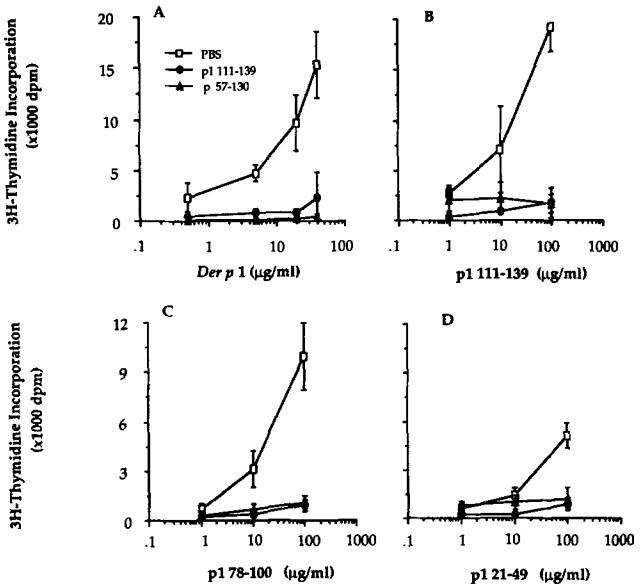

Figure 1. Peptides given intranasally to mice can inhibit T cell responses. Mice were treated with either PBS (□), 100 μg of GEX p57–130 (▲), or p1 111–139 (●) intranasally on three consecutive days and 1 wk later all mice were immunized with 100 μg of Der p 1/CFA. LN cells were collected 7 d later and cultured in vitro with (*A*) Der p 1 protein, (*B*) p1 111–139, (*C*) p1 78–100, or (*D*) p1 21–49 for 24 h. Data shows the mean IL-2 response of five mice per group ± SD.

cell proliferation were determined. LN cells from control mice proliferated strongly to the peptide in vitro and produced IL-2 in a dose-dependent manner. However, T cells from peptide-treated mice proliferated only poorly and secreted only very low levels of IL-2 when rechallenged with the peptide in vitro (data not shown). Thus the induction of tolerance results in a state of nonresponsiveness to the whole antigen as a result of the failure of antigen-specific T cells to secrete IL-2 and to proliferate when restimulated. Tolerance to the immunodominant epitope also downregulated the response of T cells directed to other epitopes on the antigen. Recently it has been reported that susceptible $H2^u$ mice are protected from developing EAE by inhalation of a synthetic peptide containing the immunodominant epitope MBP Ac 1–9 or Ac 1–11 before encephalitogenic challenge with MBP or peptide (16). In this study (16), the authors also observed that after the induction of tolerance to Ac 1–9 T cell responses to subdominant epitopes on MBP were equally downregulated.

To determine the concentration of peptide required to induce peripheral tolerance, mice were treated intranasally on three consecutive days with either PBS or 1, 10, or 100 μg of p1 111–139 and then challenged 1 wk later with Der p 1 in CFA. LN cells were collected 7 d after immunization and cultured in vitro with either Der p 1 or p1 111–139. T cell responses from each of the test groups after in vitro stimulation with Der p 1 or p1 111–139 were markedly downregulated (Table 1), whereas T cells from control mice secreted large amounts of IL-2 when stimulated in vitro with either Der p 1 or peptide (Table 1). The immunodominant peptide at concentrations as low as 3 × 1 μg induced peripheral tolerance when given intranasally. It is interesting to note that inhalation of similar levels of an intact protein in mice can induce immunological tolerance (12). Therefore mucosa of the respiratory tract appears to provide a highly efficient route for the induction of tolerance.

Mice tolerized with p1 111–139 also failed to produce specific Abs. To demonstrate this, spleen cells collected from Der p 1 immune mice were cultured in vitro either alone or with LN cells from mice that had been treated intranasally with PBS or p1 111–139 and immunized subcutaneously with Der p 1 in CFA. Lymphocytes from both the test and control groups were cultured with Der p 1 for 7 d and the presence of anti-Der p 1 Abs in the culture supernatants were examined by ELISA. Der p 1-specific Abs were present in the supernatants of both immune spleen cells and LN cells from PBS-treated mice, but no Abs were detected in the superna-

Table 1. *Intranasal Tolerance Induced with Microgram Quantities of Peptide*

	Intranasal dose of antigen			
	PBS	3 × 1 μg	3 × 10 μg	3 × 100 μg
In vitro antigen				
Der p 1 (40 μg/ml)	5,075 (± 1,354)	890 (± 314)	1,088 (± 391)	827 (± 321)
p1 111-139 (100 μg/ml)	10,698 (± 3,477)	1,671 (± 692)	999 (± 643)	773 (± 537)
Medium	241 (± 62)	328 (± 139)	560 (± 298)	401 (± 537)

C57BL/6J mice were treated on three consecutive days with p1 111–139 intranasally with the doses indicated or with PBS. 1 wk later mice were immunized with Der p 1 in CFA and the periaortic and inguinal LN were collected 7 d later and cultured in vitro with either Der p 1 or p1 111–139. Data shows the mean response of five mice per group ± SD.

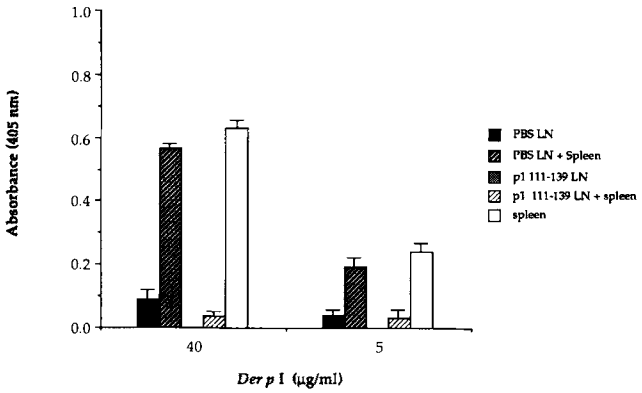

Figure 2. Inhibition of antibody production in vitro. Spleens were removed from Der p 1 immune mice and the periaortic and inguinal LN were collected from control or tolerized mice who had been immunized with Der p 1/CFA. 4×10^5 spleen or LN cells were cultured alone or 2×10^5 spleen cells were mixed with equal numbers of LN cells from either control or tolerized mice. All cells were cultured for 7 d at 37°C and supernatants were assayed for Der p 1-specific Abs using an ELISA assay. Data show the mean response from five mice per group and is presented as ELISA OD units at 405 nm.

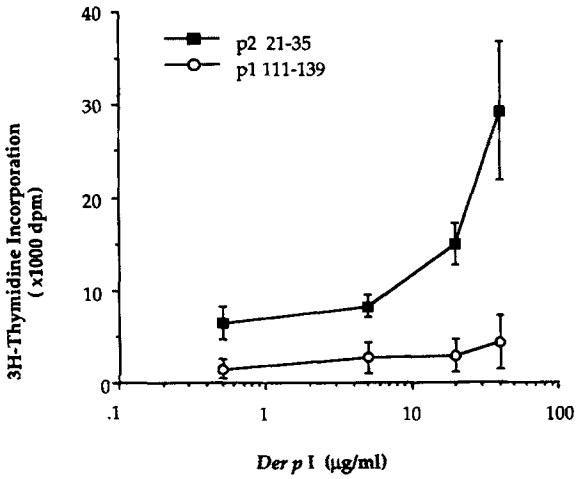

Figure 3. Peptide therapy inhibits ongoing immune responses to the Der p 1 allergen. Mice were treated on five consecutive days with p1 111–139 (○) or a control peptide (■) 10 d after sensitization with Der p 1. 1 wk after the completion of therapy mice were reimmunized with Der p 1/IFA and LN cells were collected 7 d later. The data shows the mean IL-2 response of individual mice ± SD. The number of animals per group used in each experiment were control (*p2 21–35*) $n = 5$, and tolerized (*p1 111–139*) $n = 10$. This experiment has been repeated with over 20 animals with equivalent results.

tants of LN cells from mice tolerized with p1 111–139. When LN cells from PBS-treated mice were mixed with immune spleen cells, Ab production was unaffected (Fig. 2). However, addition of LN cells from p1 111–139-treated mice to spleen cell cultures completely inhibited antibody production in vitro (Fig. 2). The mechanisms which lead to inhibition of antibody synthesis have all the hallmarks of being classical suppression. That is, not only do tolerized T cells fail to provide cognate help to primed B cells in vitro, but the tolerized cells also have the capacity to suppress the effector function of primed $CD4^+$ Th cells in the spleen.

Since allergy to HDM reflects a chronic disease, we wished to examine whether it were possible to inhibit an on-going T cell response using intranasal peptide therapy (16). Mice were immunized with Der p 1 in CFA and 10 d later they received either 100 μg of p1 111–139 or an irrelevant peptide from the Der p 2 allergen, p2 21–35, intranasally on five consecutive days. After 7 d the mice were reimmunized with Der p 1 in IFA. 1 wk later, LN cells were cultured in vitro with the Der p 1 protein and IL-2 production was measured in supernatants of 24-h cultures. LN cells from control mice treated with the nonimmunogenic peptide p2 21–35 were able to produce IL-2 responses when stimulated with the allergen in vitro (Fig. 3), whereas IL-2 secretion was completely abrogated in LN cells from mice receiving p1 111–139 (Fig. 3). Equivalent results were obtained if mice were immunized with Der p 1/CFA and then 6 mo later received p1 111–139 on five consecutive days and then were rechallenged with Der p 1/IFA 7 d after therapy (Fig. 4). Therefore intranasal peptide therapy will induce tolerance not only in naive mice but also will inhibit the function of T cells previously sensitized to the antigen.

The studies reported here demonstrate that inhalation of peptides in low concentrations will induce peripheral tolerance in mice such that they become profoundly unresponsive to a powerful subcutaneous challenge with the antigen. Peptides containing a dominant T cell epitope were more effective tolerogens than those containing only minor epitopes, since tolerance could be induced in naive mice with only one exposure to p1 111–139, whereas a minimum of three doses were required to induce tolerance to a peptide containing a minor epitope (p1 156–168) (data not shown). Furthermore,

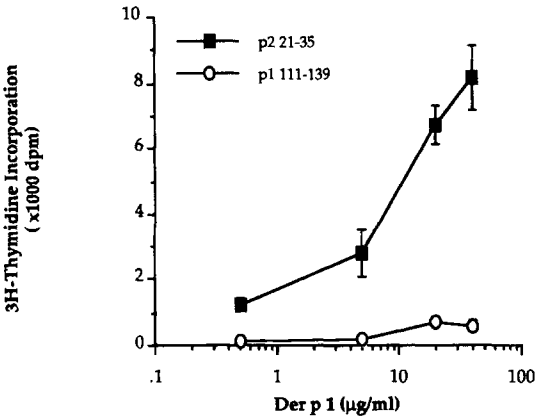

Figure 4. Inhibition of long-term immune responses to Der p 1. C57BL/6J mice were immunized with 100 μg of Der p 1/CFA i.p. and 3 wk later mice were rechallenged with 100 μg of Der p 1/IFA i.p. 6 mo later, half the mice were given 100 μg of p1 111–139 intranasally for 5 d, whereas the other half received p2 21-35 over the same period, and 1 wk later all mice were immunized with 100 μg Der p 1/CFA s.c. at the base of tail and LN cells were cultured 1 wk later. Data shows the mean IL-2 response of five mice per group ± SD.

we found that intranasal peptide therapy does inhibit an ongoing immune response in mice previously sensitized to the HDM allergen. Clearly these results have important implications showing that peptides could be effective immunotherapeutic agents in allergic conditions.

It should be emphasized from these studies that although only one peptide epitope was used for treatment, T cell responses to the other epitopes of the antigen were subsequently downregulated. This spreading of tolerance to encompass all epitopes of a protein antigen shares some similarities to the model of "infectious" tolerance (21). However, we cannot exclude other regulatory mechanisms operating within the lung that may be active in modulating the response antigen-reactive T cells. There is precedence in the literature for a role of $CD8^+$ T suppressor cells in immunological tolerance after inhalation of OVA in rats (13). Therefore, there maybe more than one mechanism operating to control T cell responses to foreign antigens encountered within the nasal mucosa and the lung. Finally, the HDM response in mice provides a promising model for studying further the peptide-induced modulation of allergen-reactive T cells.

We thank Dr. B. A. Askonas for helpful discussions and for carefully reviewing the manuscript.

This work was supported by grants from the Wellcome Trust, the Asthma Foundation of Western Australia, and the National Health and Medical Research Council of Australia. Robyn O'Hehir is the recipient of a Wellcome Senior Research Fellowship in Clinical Sciences.

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Received for publication 20 July 1993.

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