

Regulation of house dust mite responses by intranasally administered peptide: transient activation of CD4⁺ T cells precedes the development of tolerance *in vivo*

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Keywords: allergy, immunotherapy, T cell cytokines, tolerance

Abstract

We have previously demonstrated that intranasal (i.n.) administration of an immunodominant peptide (p1 111–139) derived from the house dust mite (HDM) allergen Der p 1 inhibits antigen-specific CD4⁺ T cell responses in H-2^b mice. Here we report that i.n. peptide induced a rapid but transient activation of MHC class II restricted CD4⁺ T cells that peaked 4 days after peptide treatment and was of similar magnitude to that induced by parenteral immunization with antigen in adjuvant. During the early phase of the response lymph node and splenic T cells secreted a range of lymphokines when re-stimulated *in vitro* with p1 111–139; however, by day 14 IL-2 and IFN- γ secretion by T cells were down-regulated. Mice deficient in CD8⁺ T cells became tolerant by i.n. treatment with peptide, suggesting that CD8⁺ T cells are not involved in down-regulating the CD4⁺ T cell response. Re-challenging mice with a single dose of p1 111–139 21 days after the initial treatment elicited a further transient T cell response, which was subsequently down-regulated over time. Although the i.n. peptide induced a strong transient CD4⁺ T cell response, only low levels of peptide-specific antibodies were detected either after the initial or subsequent i.n. exposures to p1 111–139. Our findings address the mechanisms underlying peripheral T cell tolerance following i.n. administration of a high dose of immunogenic peptide and have implications for understanding the consequences of peptide immunotherapy.

Introduction

Antigen-specific CD4⁺ T cells play an important role in allergic sensitization by secreting lymphokines that promote IgE synthesis and support the growth or maturation of effector cells such as eosinophils and mast cells (1). Protein antigens derived from the house dust mite (HDM), *Dermatophagoides pteronyssinus* are a common source of aeroallergens in the environment, ~10% of the population develop allergies to HDM resulting in clinical symptoms including perennial rhinitis, atopic dermatitis or asthma (1). At present desensitization therapy is not successful for most HDM allergic patients and more effective methods of modulating the allergic immune response are required. Recently it has been shown that human HDM-reactive CD4⁺ T cells can be inactivated *in vitro* by exposing them to supraoptimal concentrations of their

cognate peptide (2,3). Furthermore, it is possible to inhibit the function of allergen-reactive T cells *in vivo* by either intranasal (i.n.) or oral administration of a peptide containing the immunodominant T cell epitope derived from the HDM allergen Der p 1 in H-2^b mice (4,5). These results suggest that peptide-mediated immune regulation may be useful in allergen immunotherapy as has been demonstrated in the prevention of experimental autoimmune diseases (6–9).

Administration of antigens via mucosal surfaces is an effective way of inhibiting antigen-specific T cell responses *in vivo*. Oral tolerance to protein antigens results in the inhibition of antigen-specific CD4⁺ T cell responses *in vitro* by either clonal deletion, clonal anergy or active suppression depending on the dose of antigen administered (10,11).

Little is known about the mechanisms that regulate immune responses following high-dose antigen treatment through the respiratory tract. In rats continuously exposed to low doses of nebulized protein, CD4⁺ T cells become primed to the antigen and secrete IL-2 and IL-4 (12). The CD4⁺ T cell response, however, is then suppressed by the activation of IFN- γ -secreting CD8⁺ T cells and these suppressor cells abrogate the induction of IgE synthesis in naive recipients upon adoptive transfer (12–14). In this paper we have begun to elucidate the cellular mechanisms which give rise to the development of antigen-specific T cell tolerance following i.n. administration of peptide. We examined the nature of the primary T cell response elicited by high doses of i.n. peptide and investigated the role of CD8⁺ T cells in the induction of tolerance. We also demonstrate that rechallenging mice with i.n. peptide after 3 weeks after the initial treatment elicits a further transient T cell response to the peptide. These studies have important implications in understanding the response to mucosally delivered peptides in immunotherapy.

Methods

Animals

Inbred female C57BL/6J mice were purchased from Harlan OLAC (Bicester, UK) at 6–8 weeks of age and were kept in isolators. CD8^{-/-} mice were obtained as a fifth generation backcross on the C57BL/6J background and bred under conventional conditions. The mice were kindly provided by Dr Dimitri Kioussis (National Institute of Medical Research, The Ridgeway, Mill Hill, UK).

Antigens

The synthetic peptide p1 111–139 was derived from the Der p 1 sequence and was synthesized using standard f-moc chemistry.

Antibodies

Monoclonal antibodies specific for murine CD4 (YTS.191.2.1) and CD8 (Lyt2, 53.5.) were obtained from the European Tissue Culture Collections. The murine anti-I-A^b mAb (M5/114) was a kind gift from Dr R. Lechler. Cervical lymph node (LN) cells from peptide-treated mice were cultured with mAb supernatants or a control rat anti-mouse IgG at 1/20 and 1/100 dilution in the presence or absence of peptide for 24 h at 37°C. Supernatants were collected and assayed for the presence of lymphokines.

Induction of T cell non-responsiveness by inhalation of peptide

Mice were lightly anaesthetized with ether and the peptide p1 111–139 (100 μ g) dissolved in 20 μ l of PBS was administered i.n. using a micropipette on three consecutive days. Mice were immunized s.c. at the base of the tail 14 days after the last treatment with 50 μ g of Der p 1 emulsified in complete Freund's adjuvant (CFA), (Difco, Detroit, MI) in a volume of 0.2 ml.

Culture medium

LN or spleen cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2% FCS

(Gibco), 50 μ M 2-mercaptoethanol (Sigma, St Louis, MO), 2 mM L-glutamine (Sigma) and 20 μ g/ml penicillin/streptomycin. CTLL-2 cells were maintained in RPMI 1640 (Gibco) and 10% FCS, while FDC-P1 cells were cultured in DMEM plus 5% FCS.

T cell assays

The cervical LNs were pressed through a stainless steel wire mesh, washed and cultured at 4×10^5 cells in 0.2 ml culture medium in 96-well flat-bottom tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at 37°C. Protein or peptide antigen was added at various concentrations and supernatants were collected at 24 or 48 h and stored at -20°C until assayed.

Lymphokine assays

The CTLL-2 cell line proliferates maximally with IL-2 but only poorly in the presence of IL-4 (15). Test supernatants (50 μ l) were added in triplicate to 5×10^3 CTLL-2 cells (50 μ l) per well and cultured for 24 h at 37°C. Tritiated methyl thymidine (³[H]TdR; 1 μ Ci, Amersham, Amersham, UK) was added during the last 6 h of culture.

FDC-P1 cells proliferate in response to IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) (15). FDC-P1 cells (2×10^3 in 50 μ l) were cultured with the test supernatants in triplicate wells for 40–48 h and then pulsed with 1 μ Ci ³[H]TdR for the last 6 h.

CT.4S cells proliferate in response to IL-4, but only poorly to IL-2. Cells (2×10^3) were cultured with test supernatants for 96 h and pulsed with ³[H]TdR for 6 h. The level of radioactivity incorporated by lymphokine dependent cell lines was determined by harvesting cells onto glass fibre filter mats and counted using a Beta Plate Liquid Scintillation counter (Wallac).

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

Antibody measurements

The p1 111–139 peptide was coated onto Immulon-4 microtitre plates at 10 μ g/ml overnight at 4°C. Plates were blocked with PBS containing 10% BSA. After washing, serum dilutions were added to the plate and incubated for 2 h at room temperature. Plates were washed and the presence of bound antibody was detected isotype-specific biotinylated antibodies (Southern Biotechnology Associates). Plates were washed and developed using streptavidin-alkaline peroxidase (Sigma) with 3,3'-5,5'-tetramethyl benzidine (Sigma) as the substrate. Results expressed as OD absorbance units at 405 nm.

Results

T cells undergo transient activation during the induction phase of non-responsiveness

In order to investigate the effect of i.n. peptide on the effector function of antigen-specific T cells *in vivo*, C57BL/6J mice were treated i.n. on three consecutive days with 100 µg of p1 111–139. This regime has previously been shown to be effective in inducing peripheral tolerance to Der p 1 in naive mice. The response of T cells in the draining cervical LN and spleen of i.n. treated mice was examined at various times over a 28 day period and their capacity to secrete lymphokines following antigenic challenge *in vitro* was determined. When T cells are re-stimulated with peptide *in vitro* on day 2 they secrete high levels of IL-3/GM-CSF but only low levels of IFN-γ and IL-2 (Fig. 1). By day 4, p1 111–139-specific T cells in the LN and spleen display their highest level of secretion of IL-2, IL-3/GM-CSF and IFN-γ (Fig. 1) with little or no IL-4 (not shown). By day 8 T cell responses in the cervical LN and spleen had begun to decline, and on day 14 and day 28 peptide-specific T cells secreted only low levels of IL-3/GM-CSF and no IL-2 or IFN-γ when stimulated *in vitro* (Fig. 1). During the first 4 days after peptide inhalation there was a

rapid increase in the cellularity of the cervical LN that declined over time (Fig. 1d). Spleen cell numbers, however, did not change significantly over the same period. Thus it would appear that by day 14 antigen-specific T cells were anergic as defined by their inability to secrete IL-2 when stimulated *in vitro*.

Identification of the phenotype of the cell responding to peptide

It was important to identify the phenotype of the T cells which responded to the i.n. peptide. Mice were treated i.n. with 100 µg of p1 111–139 on three consecutive days and 4 days later the cervical LN were cultured with 10 µg/ml p1 111–139 either in the presence or absence of mAbs against CD4, CD8, class II MHC or a control antibody (rat anti-IgG). Cells treated with the control antibody responded strongly to the peptide *in vitro* secreting IL-2, IL-3/GM-CSF (Fig. 2) and IFN-γ (not shown). Treating cells with a 1/20 dilution of anti-CD4 mAb inhibited lymphokine secretion in response to the peptide, while anti-CD8 antibody had no effect (Fig. 2). Treating cells with an antibody to class II MHC (anti-I-A^b) also abrogated the secretion of lymphokines in response to peptide stimulation *in vitro* (Fig. 2). These results indicate that the cells responding

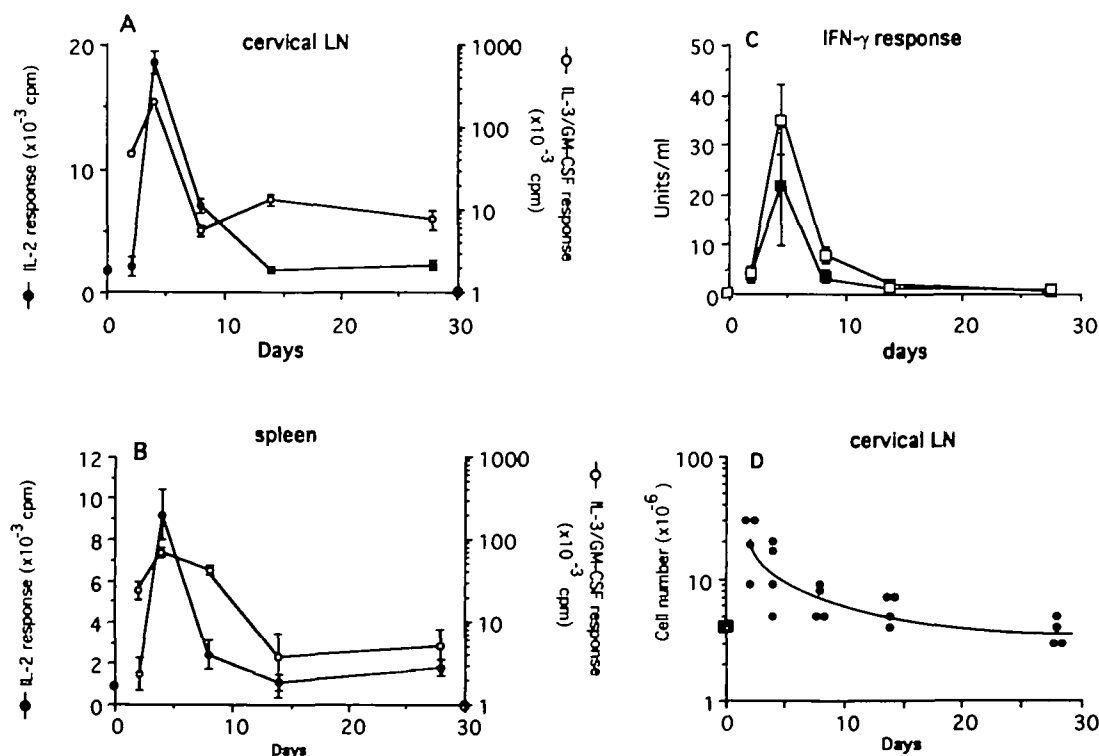


Fig. 1. Lymphokine production by T cells following i.n. administration of peptide. Mice were treated with 100 µg p1 111–139 on three consecutive days, and (A) the cervical LN and (B) spleen were cultured *in vitro* on days 2, 4, 8, 14 and 28 post-i.n. treatment and stimulated with the peptide. (A and B) Supernatants were collected and assayed for the presence of IL-3/GM-CSF (○) and IL-2 (●). (C) Cervical LN (□) and spleen (■) cells were cultured *in vitro* with p1 111–139 and the secretion of IFN-γ was detected in 48 h supernatants. The data show the mean response of five mice per time point $\pm$ SD. The data presented is representative of three separate experiments. LN or spleen cells in these experiments were cultured in the presence of varying concentrations of p1 111–139, but only results for 10 µg/ml are shown since this is the dose of peptide that gives optimal T cell activation *in vitro*. Responses from naive T cells stimulated with p1 111–139 (10 µg/ml) on day 0 are usually <1000 c.p.m. for IL-2 and <800 c.p.m. for IL-3/GM-CSF. (D) Data show the average cell yields from the cervical LN and is from one representative experiment.

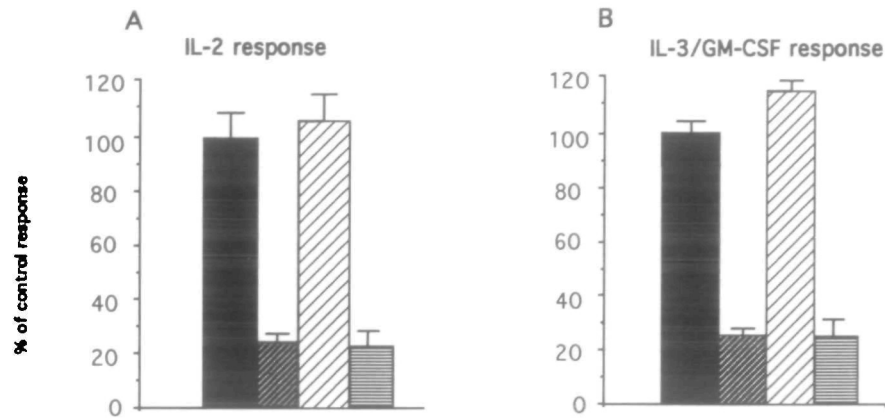


Fig. 2. CD4⁺ T cells respond to the i.n. peptide. Mice were treated with p1 111–139 i.n. and 4 days later the cervical LN were cultured *in vitro* with the peptide at 10 µg/ml. Cells were treated with a control rat anti-IgG (■) or mAbs against either CD4 (■), CD8 (▨) or I-A^b (▤) (see Methods). Supernatants were then assayed for the presence of IL-3/GM-CSF or IL-2. Results are expressed as mean response of five mice $\pm$ SD.

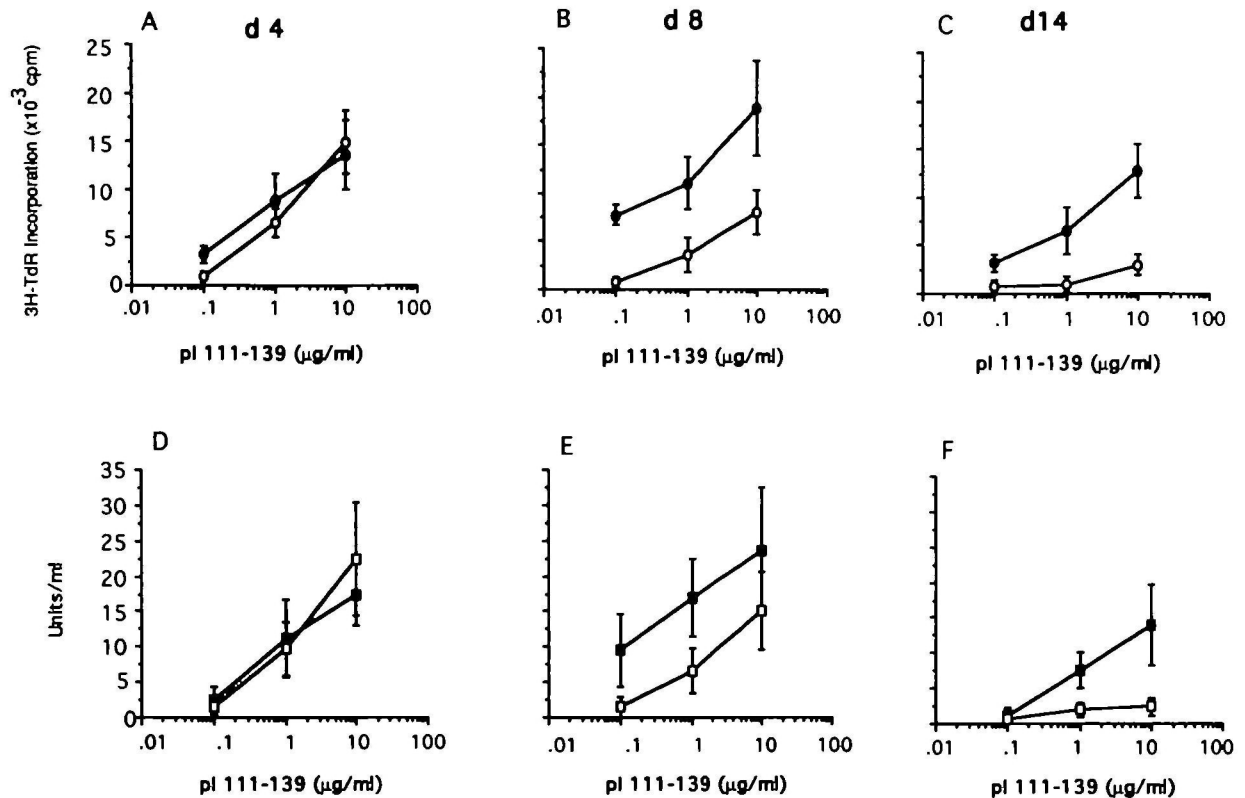


Fig. 3. The induction of tolerance leads to an equivalent level of T cell activation. Mice were treated with 3×100 µg of p1 111–139 i.n. or 50 µg Der p 1/CFA s.c. at the scruff of the neck and the cervical LN (■) and spleen (□) cell responses were examined *in vitro* 4, 8 and 14 days after the final challenge. Data show the mean (A–C) IL-2 and (D–F) IFN-γ response from five mice per time point $\pm$ SD.

to high-dose HDM peptide are class II MHC restricted and express CD4 co-receptor.

Comparison of antigen-specific T cell responses following an activating or tolerizing challenge with antigen

Mice were treated i.n. with 3×100 µg of p1 111–139 or were immunized at the scruff of the neck with 50 µg Der p 1/CFA.

T cell responses were measured in the cervical LN on days 4, 8 and 14 after both forms of challenge, and production of IL-2 and IFN-γ was examined. On day 4 there was little difference in the T cell responses of mice receiving the tolerizing or activating challenges with antigen. T cells from both groups secreted equivalent levels of IL-2 and IFN-γ (Fig. 3). However, by day 8 T cells from the peptide-treated

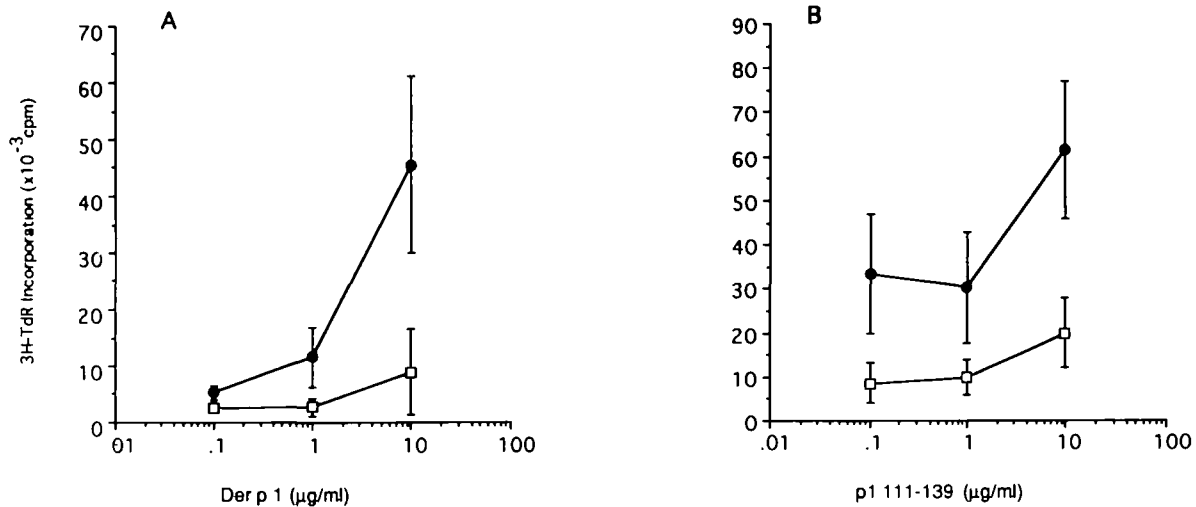


Fig. 4. CD8⁺ T cells do not play a role in tolerance to i.n. peptide. CD8^{-/-} mice were treated with either PBS or p1 111-139 i.n. in a typical tolerance experiment. At 10 days after parenteral challenge with Der p 1/CFA LN cells were cultured *in vitro* in the presence or (A) Der p 1 or (B) p1 111-139. Data show the mean IL-2 response from five mice per group ± SD.

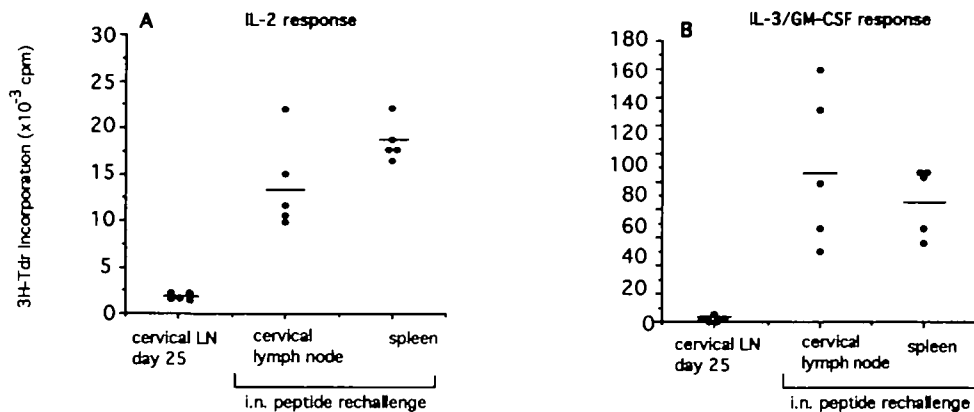


Fig. 5. Antigen-specific T cells display a further transient response after rechallenge with i.n. p1 111-139. Mice were treated with 100 μg p1 111-139 on three consecutive days and 21 days later mice were given a single 100 μg dose of p1 111-139 i.n., while other mice were left untreated. Four days post-challenge, the cervical LN and spleen cells were cultured *in vitro* with p1 111-139 and the 24 h supernatants were assayed for the presence of (A) IL-2 and (B) IL-3/GM-CSF. Data shows the mean response of five mice per group ± SD. The data are representative of three separate experiments.

mice had begun to down-regulate their cytokine secretion, and this was most noticeable by day 14 (Fig. 3).

CD8⁺ T cells do not play a role in the maintenance of peripheral tolerance to inhaled peptide

To examine whether regulatory CD8⁺ T cells play a role in inhibiting the function of antigen-specific CD4⁺ T cells following i.n. peptide treatment we used mice deficient in CD8⁺ T cells. The CD8^{-/-} mice were treated with PBS or with p1 111-139 i.n. using the standard tolerance protocol. Following Der p 1 immunization, LN cells from PBS treated animals were still responsive and could secrete IL-2 when stimulated *in vitro* with either Der p 1 or the dominant peptide (Fig. 4). CD8^{-/-} mice that had been pretreated with p1 111-139 i.n. had down-

regulated antigen-specific T cell responses to both the protein and the peptide, typical of wild-type mice (Fig. 4).

Intranasal peptide challenge in vivo after anergy induction results in further transient antigen-specific IL-2 production

Twenty-one days after i.n. peptide, antigen-specific T cells in the regional LN and spleen fail to secrete IL-2 when stimulated *in vitro* with p1 111-139. Thus we wished to examine if peptide-specific T cells would remain anergic after a further i.n. challenge with p1 111-139. Four days after the *in vivo* rechallenge, T cells from the cervical LN and spleen secreted IL-2 and IL-3/GM-CSF in an antigen-specific manner when stimulated *in vitro* with peptide (Fig. 5). In comparison, T cells from unchallenged mice failed to secrete IL-2 and only low

levels of IL-3/GM-CSF (Fig. 5). However, the secretion of IL-2 by peptide-specific T cells was only transient, and by day 14, T cells had again down-regulated IL-2 production. It should be noted that despite the transient release of IL-2 from T cells upon rechallenge with peptide *in vivo* mice remained unresponsive to a challenge with Der p 1/CFA.

We were interested in determining if the same effect would be seen after parenteral challenge with Der p 1. Mice were treated with PBS or p1 111–139 and challenged 2 weeks later with Der p 1/CFA s.c. at the base of tail. LN cells were collected either on day 4 or 10 post-challenge and examined for their capacity to secrete IL-2 following *in vitro* stimulation with p1 111–139. T cells from control mice could secrete

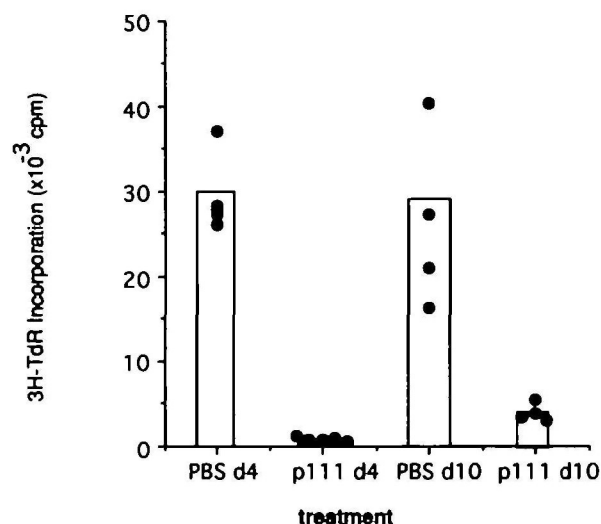


Fig. 6. Intranasally treated mice remain unresponsive following challenge with HDM protein. Mice were treated with PBS or p1 111–139 and immunized 2 weeks later with Der p 1/CFA. LN cells were tested for their capacity to secrete IL-2 on days 4 and 10 post-challenge. The results show the 24 h IL-2 response from T cells of individual mice.

IL-2 at high levels on both days 4 and 10 post-challenge (Fig. 6), whereas on the other hand T cells from peptide-treated mice failed to secrete IL-2 at either time point (Fig. 6).

Peptide inhalation does not elicit a significant antibody response

Since inhalation of peptide induced a vigorous T cell response we wanted to determine if there were any serum antibodies produced to the peptide. Mice were treated with 3×100 µg p1 111–139 i.n. and bled on day 14 after the last treatment (Group 1, Fig. 7). The level of peptide-specific antibodies in the serum was low and predominantly of the IgM isotype with a small amount of IgG2a (Fig. 7). Unlike the T cell response we did not observe any significant rise either in the level of peptide-specific antibodies or a switch to the IgG isotype in mice who were rechallenged twice with p1 111–139 i.n. at fortnightly intervals (Group 2, Fig. 7).

Discussion

We report here that i.n. administration of a high dose of HDM-derived peptide elicits a strong but transient activation of CD4⁺ T cells that eventually gives rise to a state of antigen-specific T cell non-responsiveness. Responses to the i.n. peptide spread systemically into various lymphoid tissues within 48 h, but are strongest in the draining cervical LN. The peptide elicited a vigorous response *in vivo* as indicated by a 3- to 4-fold increase in the cellularity of the cervical LN over the first 4 days. The T cell responses peaked on day 4 after the completion of treatment, at which time the cells were capable of secreting IL-2, IL-3/GM-CSF and IFN-γ but with little or no IL-4 when stimulated with p1 111–139 *in vitro*. By 2 weeks, peptide-specific T cells had down-regulated IL-2 and IFN-γ secretion but there was residual antigen-specific secretion of IL-3/GM-CSF responses even on day 28. In addition we noticed that the activation of T cells following peptide inhalation appeared to be of the same magnitude as that elicited by a conventional immunization with Der p 1 in CFA.

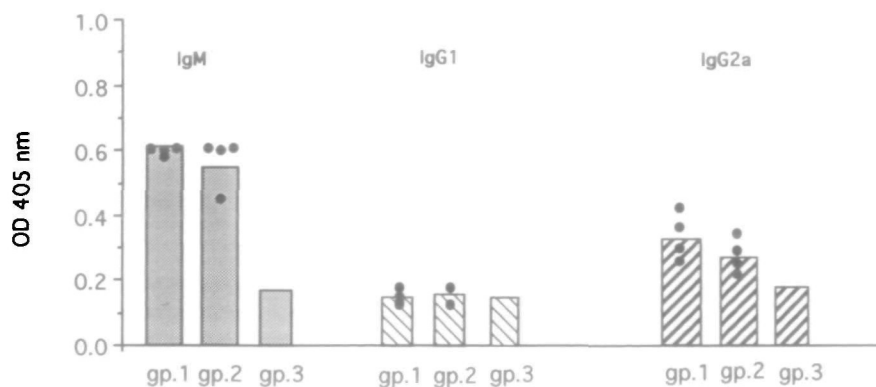


Fig. 7. Intranasally administered peptide induces minimal antibody responses *in vivo*. Group 1 mice were treated with 100 µg p1 111–139 i.n. on three consecutive days and 14 days later were bled. Group 2 mice received the same initial treatment but also were rechallenged with p1 111–139 on days 17 and 31, and were bled on day 45. The serum from individual mice of both groups was analysed for the presence of IgM, IgG1 and IgG2a peptide-specific antibodies by ELISA. Group 3 refers to control responses obtained with normal mouse serum.

The failure of T cells to secrete IL-2 following antigenic stimulation has been used as a definition for clonal anergy (16). However, from our studies it would appear that the development of anergy *in vivo* occurring as a consequence of i.n. peptide is mediated by a different mechanism to that proposed for anergy *in vitro* (17,18). After peptide i.n. CD4⁺ T cells still secrete IL-2, and therefore, must have received costimulation from antigen-presenting cells (APCs). Nevertheless, the signals which mucosal APCs deliver to the naive T cells must in some way prime these cells to become non-responsive (anergic). Studies to date have suggested that mucosal APCs do not have an intrinsic tolerogenic capacity (19). Whether or not certain populations of APCs are able to deliver inhibitory signals that override the known positive costimulatory signals such as those mediated by the B7 family of molecules is not known.

Our findings are consistent with previous studies which have also demonstrated that CD4⁺ or CD8⁺ T cells responding to various tolerogens undergo a state of transient activation prior to the development of non-responsiveness (20–24). However, we have obtained no direct evidence for a shift in cytokine production by CD4⁺ T cells over time following the induction of tolerance to i.n. peptide. This is in contrast to recent findings which show that the development of peripheral CD4⁺ T cell tolerance following immunization of mice with ovalbumin in incomplete Freund's adjuvant (25). Others suggest a role of CD8⁺ T_H cells in the regulation of IgE responses in mice and rats exposed to nebulized antigen (13,14,26). However, we failed to demonstrate that CD8⁺ T cells regulate either the induction or maintenance of tolerance to peptide administered i.n., similar to that described for the induction of oral tolerance (27). There is a possibility that CD8⁺ T_H cells may function when low doses of antigen are encountered by the immune system, such as in the maintenance of oral tolerance induced by feeding low doses of protein antigen (10). It should be noted that we have only examined responses to high doses of peptide given i.n., which may occur via distinct mechanisms.

Two weeks after peptide treatment, T cells in the draining cervical LN failed to secrete IL-2 when challenged *in vitro*. However, if mice were challenged 21 days after their initial treatment with a single 100 µg dose of peptide i.n., then within 4 days of the challenge, antigen-specific T cells capable of secreting IL-2 were detected in the cervical LN and spleen. The capacity of these T cells to secrete IL-2 was only transient and was down-regulated by 2 weeks. It would appear that IL-2 production on peptide rechallenge *in vivo* did not abrogate peripheral tolerance to the HDM protein, since these same animals were still unresponsive to a parenteral challenge with Der p 1/CFA if given 14 days after the i.n. rechallenge. Furthermore, if mice are continuously rechallenged with peptide i.n. once every 2 weeks then by day 4 after the last rechallenge the presence of IL-2 secreting cells in the cervical LN and spleen is observed. Similar responses have also been shown to occur with periodic feeding of high doses of antigen to mice (28) and with tolerance to the superantigen staphylococcal enterotoxin B (SEB) (29). Following the induction of peripheral tolerance to SEB V β 8⁺ CD4⁺ and CD8⁺ T cells appear to be anergic when stimulated with antigen *in vitro*. However, on rechallenge with SEB *in vivo* V β 8⁺ TCR⁺

T cells regain the capacity to secrete IL-2 but only CD8⁺ T cells were shown to undergo clonal expansion (29). It is unclear at this stage why the transient IL-2 response is not observed following parenteral challenge with Der p 1 in peptide treated mice. This may be due to differences in epitope concentration or may involve different APCs. Despite the strong transient CD4⁺ T cell response that occurs to the i.n. peptide these cells fail to provide effector help to B cells to induce Ig class switching. Although some IgM peptide-specific antibodies are present, no IgG antibodies can be detected in the serum of mice. Our findings suggest that the transient IL-2 response observed after peptide rechallenge is from naive T cells that have not yet been tolerized to the peptide. What is clear from these experiments is that the development of peripheral tolerance to i.n. administered, or fed, antigens is not complete after the first encounter with antigen, but instead the immune system continuously responds to these antigens in a negative way that acts to preserve tolerance.

It is possible that the development of mucosal tolerance involves more than one mechanism in order to control responses to common dietary or inhaled antigens. For example, following high-dose antigen treatment some T cells may undergo apoptosis as a result of antigen recognition (11,30), a subset of cells may escape death but become anergic (31). In addition, negative responses mediated by regulatory T_H cells may be superimposed on these effects (14,26). Therefore, different overlapping mechanisms of immune regulation at mucosal surfaces may provide for an efficient control over immune responses to normal environmental antigens.

Our results have important implications for the use of peptides in immunotherapy. Currently a clinical trial is in progress investigating the efficacy of synthetic peptides derived from the Fel d 1 allergen to ameliorate allergic symptoms in cat allergic individuals (32). Our results are likely to provide an insight into the mechanisms of how mucosally delivered peptides induce peripheral tolerance in CD4⁺ T cells *in vivo*. In addition we have demonstrated that the immune system continuously responds to the peptide tolerogen on periodic challenge *in vivo*. This is an important observation since patients undergoing peptide immunotherapy are expected to receive peptide injections every 3–4 weeks over an extended period of time. Clearly information is required to better understand the cellular and molecular basis for tolerance induction by peptides delivered through mucosal surfaces. This will require examination of the nature of mucosal APCs and the nature of the signals delivered to the T cells.

Acknowledgements

This work was supported by grants from the Wellcome Trust and the Medical Research Council, UK.

Abbreviations

APC	antigen-presenting cells
CFA	complete Freund's adjuvant

GM-CSF	granulocyte macrophage colony stimulating factor
HDM	house dust mite
i.n.	intranasal
LN	lymph node
SEB	staphylococcal enterotoxin B

References

- O'Hehir, R. E., Garman, R. D., Greenstein, J. L. and Lamb, J. R. 1991. The specificity and regulation of T cell responsiveness to allergens. *Annu. Rev. Immunol.* 9:67
- O'Hehir, R. E., Yssel, H., Verma, S., de Vries, J. E., Spits, H. and Lamb, J. R. 1991. Clonal analysis of differential lymphokine secretion in peptide and superantigen induced anergy. *Int. Immunol.* 3:819.
- Higgins, J. A., Lamb, J. R., Marsh, S. G. E., Tonks, S., Hayball, J. D., Rosen-Bronson, S., Bodmer, J. G. and O'Hehir, R. E. 1992. Peptide-induced non-responsiveness of HLA-DP restricted human T cells reactive with *Dermatophagoides* spp (house dust mite) *J. Allergy Clin. Immunol.* 90:749.
- 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 the major house dust mite allergen in naive and sensitized mice *J. Exp. Med.* 178:1783.
- Hoyne, G. F., Callow, M. G., Kuo, M.-C. and Thomas, W. R. 1994. Inhibition of T cell responses by feeding peptides containing major and cryptic epitopes. Studies with the *Der p 1* allergen. *Immunology* 83:190
- 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.
- Dick, A. D., Cheng, Y. F., McKinnon, A., Liversidge, J. and Forrester, J. V. 1993. Nasal administration of retinal antigens suppresses the inflammatory response in experimental allergic uveoretinitis. A preliminary report of intranasal induction of tolerance with retinal antigens. *Br. J. Ophthalmol.* 77:171
- Gaur, A., Wiers, B., Liu, A., Rothbard, J. and Fathman, C. G. 1992. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy *Science* 258:1491.
- Aichele, P., Kybruz, D., Ohashi, P., Odermatt, B., Zinkernagel, R. M., Hengartner, H. and Pircher, H. 1994. Peptide-induced tolerance to prevent autoimmune diabetes in a transgenic mouse model. *Proc. Natl Acad. Sci. USA* 91:444.
- Weiner, H. L., Friedman, A., Miller, A., Khoury, S. J., Al-Sabbagh, A., Santos, L., Sayegh, M., Nussenblatt, R. B., Trentham, D. E. and Hafler, D. A. 1994. Oral tolerance. immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu. Rev. Immunol.* 12:809
- Chen, Y., Inobe, J.-I., Marks, R., Gonnella, P., Kuchroo, V. K. and Weiner, H. L. 1995. Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature* 376:177
- 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 class II-restricted CD4⁺ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J. Exp. Med.* 178:889.
- McMenamin, C., Oliver, J., Girn, B., Holt, B. J., Kees, U. R., Thomas, W. R. and Holt, P. G. 1991. Regulation of T cell sensitization at epithelial surfaces in the respiratory tract: suppression of IgE responses to inhaled antigens by CD3⁺ TCR α/β ⁺ lymphocytes (putative $\gamma\delta$ T cells). *Immunology* 74:234.
- McMenamin, C., Pimm, C., McKersey, M. and Holt, P. G. 1994. Regulation of IgE responses to inhaled antigen in mice by antigen-specific $\gamma\delta$ T cells. *Science* 265:1869
- Kelso, A. 1990. Frequency analysis of lymphokine-secreting CD4⁺ and CD8⁺ T cells activated in a graft-versus-host reaction. *J. Immunol.* 145:2167
- Gajewski, T. F., Lancki, D. W., Stack, R. and Fitch, F. W. 1994. 'Anergy' of Th0 helper T lymphocytes induces a down-regulation of Th1 characteristics and a transition to Th2 phenotype *J. Exp. Med.* 179:481.
- Jenkins, M. K. and Schwartz, R. H. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo* *J. Exp. Med.* 165:302
- Jenkins, M. K., Ashwell, J. D. and Schwartz, R. H. 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J. Immunol.* 140:3324.
- Van Wilsem, E. J. G., Van Hoogstraten, I. M. W., Breve, J., Scheper, R. J. and Kraal, G. 1994. Dendritic cells of the oral mucosa and the induction of oral tolerance. A local affair *Immunology* 83:128
- Webb, S., Morris, C. and Sprent, J. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity *Cell* 63:1249.
- Rocha, B. and von Boehmer, H. 1991. Peripheral selection of the T cell repertoire. *Science* 251:1225.
- Kybruz, D., Aichele, P., Speiser, D. E., Hengartner, H., Zinkernagel, R. M. and Pircher, H. 1993. T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides *Eur. J. Immunol.* 23:1956
- Kearney, E. R., Pape, K. A., Loh, D. Y. and Jenkins, M. K. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction *in vivo* *Immunity* 1:327
- Vidard, L., Colarusso, L. J. and Benacerraf, B. 1994. Specific T cell tolerance may be preceded by a primary response *Proc. Natl Acad. Sci. USA* 91:5627.
- Vidard, L., Colarusso, L. J. and Benacerraf, B. 1995. Specific T cell tolerance may reflect selective activation of lymphokine synthesis *Proc. Natl Acad. Sci. USA* 92:2259.
- Renz, H., Lack, G., Saloga, J., Schwinzer, R., Bradley, K., Loader, J., Kupfer, A., Larsen, G. L. and Gelfand, E. W. 1994. Inhibition of IgE production and normalization of airways responsiveness by sensitized CD8 T cells in a mouse model of allergen-induced sensitization. *J. Immunol.* 152:351
- Garside, P., Steel, M., Liew, F. Y. and Mowat, A. M. 1995. CD4⁺ but not CD8⁺ T cells are required for the induction of oral tolerance *Int. Immunol.* 7:501.
- 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 feeds. *Immunology* 84:304.
- Heeg, K., Gaus, H., Griesse, D., Bendigs, S., Miethke, T. and Wagner, H. 1995. Superantigen-reactive T cells that display an anergic phenotype *in vitro* appear functional *in vivo*. *Int. Immunol.* 7:105.
- Critchfield, J. M., Racke, M. K., Zuniga-Pflucker, J. C., Cannella, B., Raine, C. S., Gorman, J. and Lenardo, M. J. 1994. T cell deletion in high dose therapy of autoimmune encephalomyelitis *Science* 263:1139.
- Whiteacre, C. C., Gienapp, I. E., Orosz, C. G. and Bitmar, D. M. 1991. Oral tolerance in experimental autoimmune encephalomyelitis III. Evidence for clonal anergy *J. Immunol.* 147:2155.
- Norman, P. S., Ohman, J. L. and Long, A. A. 1994. Early clinical experience with T cell reactive peptides from cat allergen Fel d 1. *J. Allergy Clin. Immunol.* 93:231