

Fel d 1-derived T cell peptide therapy induces recruitment of CD4⁺CD25⁺; CD4⁺ interferon- γ ⁺ T helper type 1 cells to sites of allergen-induced late-phase skin reactions in cat-allergic subjects

C. Alexander, S. Ying, A. B. Kay and M. Larché

Allergy & Clinical Immunology, Faculty of Medicine, Imperial College London, London, UK

Summary

Background Specific immunotherapy with whole allergen extracts is associated with local accumulation of IFN- γ ⁺ and CD25⁺ cells indicating recruitment of activated T-helper type 1 (Th1) and/or T regulatory cells. We have studied allergen-induced, late-phase skin biopsies before and after T cell peptide therapy for evidence of alterations in the pattern of local recruitment of Th1, T-helper type 2 (Th2) and T regulatory cells.

Objective To evaluate the effect of T cell peptide therapy on the allergen-induced cutaneous late-phase reaction.

Methods Increasing doses of synthetic Fel d 1-derived peptides were administered (by intradermal injection) to eight cat-allergic asthmatics at 14-day intervals. Twenty-four-hour skin biopsies were taken from whole cat allergen- and diluent-injected sites, before and after treatment and studied by immunohistochemistry and *in situ* hybridization.

Results Fel-d 1 peptides decreased airway hyper-responsiveness ($P = 0.02$) and inhibited the late-phase cutaneous reaction (LPCR) to whole cat allergen ($P = 0.03$). This was associated with significant increases (post- vs. pre-treatment) in the number of cutaneous CD4⁺/IFN- γ ⁺ ($P = 0.03$) and CD4⁺/CD25⁺ cells ($P = 0.04$), but not in CD4⁺/IL-10⁺ or CD4⁺/CTLA-4⁺ cells.

Conclusions Treatment with allergen-derived T cell peptides results in allergen-dependent recruitment to the skin of Th1, rather than T regulatory cells, to cutaneous late-phase reaction sites.

Keywords allergic asthma, CD25, immune deviation, peptide immunotherapy, regulatory T cell

Submitted 4 January 2004; revised 16 July 2004; accepted 11 August 2004

Introduction

Allergen-specific immunotherapy (SIT) with allergen extracts is known to be associated with a decrease in the magnitude of the allergen-induced, late-phase cutaneous reaction (LPCR) as well as alterations in the T-helper type 1/2 (Th1/Th2) cellular cytokine profile. Thus, following treatment, the expression of IL-4 and IL-5 mRNA and protein product decreased, while IFN- γ and IL-12 mRNA and protein⁺ cells increased, suggesting that this form of therapy results in a 'switch' from a Th2, to a Th1 cytokine profile [1, 2]. An increase in the numbers of CD25⁺ cells at skin sites challenged with intact allergens was also observed [1].

CD25 is expressed on activated T cells and on certain types of regulatory T cells. T regulatory cells (T_{reg}) have variable patterns of cytokine secretion, although in a number of murine and human disease models they are associated with the elaboration of IL-10/transforming growth factor- β (TGF- β) [3–7]. Both IL-10 [8–10] and TGF- β [11] are known to

exert immunosuppressive effects *in vitro* and *in vivo* and IL-10 is increased following successful SIT [12]. Regulatory T cells frequently express the CD4⁺ CD25⁺ phenotype but fail to proliferate *in vitro* following exposure to antigen. Expression of enhanced surface levels of the costimulation molecule cytotoxic T lymphocyte-associated antigen (CTLA)-4 has also been reported to be a characteristic of regulatory cells in certain models [13]. Two studies, one with venom immunotherapy [14], and one with grass pollen [15], have shown that desensitization treatment was associated with allergen-induced recruitment of IL-10⁺ cells, consistent with the view that this form of treatment may be associated with local recruitment of T_{reg}.

The usefulness of SIT is limited by unpredictable anaphylactic reactions because of IgE crosslinking. Short, allergen-derived peptides have been evaluated as a potential therapeutic approach to atopic allergic diseases since they have the potential to inhibit T cell function but not induce anaphylaxis, because of the loss of three-dimensional conformational determinants [16–18]. In a recent double-blind, placebo-controlled study [19] we showed that overlapping peptides from Fel d 1 inhibited both the early- and late-phase skin reactions to whole cat allergen and improved

Correspondence: Mark Larché, Allergy & Clinical Immunology, Faculty of Medicine, Imperial College London, Dovehouse Street, London SW3 6LY, UK. E-mail: m.larche@imperial.ac.uk

toleration of cats. This was associated with a decrease in IL-4, IL-13 and IFN- γ production from peripheral blood mononuclear cell (PBMC) cultures. In the same cultures, increased production of IL-10 was observed.

Thus, based on previous studies of treatment with intact allergen [14, 15] or peptides [18, 19], we hypothesized that peptide immunotherapy would be associated with recruitment of CD4⁺/CD25⁺ cells, expressing either Th1 or regulatory cytokines, to sites of cutaneous allergen challenge.

Materials and methods

Human subjects

Eight cat-allergic asthmatic volunteers were recruited from the Allergy Clinic, Royal Brompton Hospital, London and by advertisement. The study was approved by the Royal Brompton and Harefield Hospitals NHS Trust Ethics Committee and informed consent was obtained from all patients. Subjects were non-smokers with no other significant illness and a normal full blood count and blood biochemical profile. Inhaled corticosteroids were discontinued at least 2 days before assessment and each study day. No volunteer had received oral corticosteroids previously. Volunteers were specifically instructed not to take antihistamines, leukotriene receptor antagonists or long-acting β_2 -agonists for 2 days prior to each study day. Volunteers had a history of wheezing on exposure to cats and demonstrated airway hyper-responsiveness (AHR), with a provocative concentration (PC₂₀) <4 mg/mL histamine, and >20% reversibility with inhaled β_2 -agonist. Cat allergy was confirmed with a positive RAST (>0.34 IU/mL; Pharmacia, Uppsala, Sweden) and a skin prick test to whole cat dander (>5 mm, ALK, Horsholm, Denmark). The histamine PC₂₀ was measured by a modification of the basic technique described by Cockcroft and colleagues [20].

Study design, peptide synthesis and safety

Eight cat-allergic, asthmatic patients (four females/four males, aged 28–46 (mean 40 years), three taking inhaled corticosteroids, four were cat owners, with a mean cat RAST of 30.3 (1.01–83.3) and histamine PC₂₀ 0.25–3.89 (mean 1.69)), were recruited and entered into an open study. Fel d 1 peptides were administered by intradermal injection in the volar aspect of the forearm. The effects of peptide administration on the cutaneous response to whole allergen challenge and on bronchial hyper-reactivity measured by histamine PC₂₀ were assessed before and 4 weeks after therapy. Skin biopsies were taken 24 h after cutaneous cat dander challenge (intradermal injection of 10 biological units (BU, ALK) containing 1.5 ng Fel d 1 in 0.01 mL), or diluent (ALK diluent, 0.3 mg/mL human serum albumin and 4 mg/mL phenol). Local skin reactions were observed after 15 min (early phase) and 24 h (late phase). Overlapping peptides from chains 1 and 2 of Fel d 1 were synthesized by F-moc chemistry at the Advanced Biotechnology Centre (Imperial College School of Medicine, London, UK). Mixing and dispensing of the peptides under sterile conditions for single-patient use were performed at Nova Laboratories Ltd (Leicester, UK).

The sequences were: Chain 1: EICPAVKRDVDLFLTGT; EQVAQYKALPVLLENA; KALPVLLENARILKNCV; RILKNCVDAKMTEEDKE; KMTEEDKENALSLLDK; KENALSVDLKIYTSPL. Chain 2: LTKVNATEPERTAMKK; TAMKKIQDCYVENGLI; SRVLDGLVMTTISSSK; ISSSK-DCMGEAVQNTV; AVQNTVEDLKLNTLGR. Incremental doses of Fel d 1 peptides (0.1, 1, 5, 10, 25 μ g; amount of each peptide in an 11-peptide mixture) were given at two-weekly intervals to reduce the incidence of peptide-induced late-phase reactions during induction of hyporesponsiveness [21, 22]. The forced expiratory volume in 1 s (FEV₁) was recorded at 5, 15, and 30 min and hourly for 6 h after each dose of peptides and after the initial whole cat dander allergen challenge (control day). A late-phase asthmatic reaction is defined as a reduction in FEV₁ of >20% below baseline measured between 1 and 6 h from peptide administration. There were no late-asthmatic reactions in any of the patients studied, at any of the doses administered. The greatest percentage decreases in the FEV₁ (mean and standard error) below baseline during the observation period was diluent: –3.1 (2.1), 0.1 μ g: –5.8 (2.6), 1 μ g: –3.0 (3.1), 5 μ g: –4.0 (1.9), 10 μ g: –3.6 (0.7), 25 μ g: –3.4 (2.6).

Intradermal testing and skin biopsies

All intradermal skin tests were performed between 08:00 and 09:00 hours. Reaction sizes were measured at 15 min and 24 h, and biopsies from each of the diluent and cat allergen-challenged site were taken at 24 h. The biopsies were processed and fixed as previously described [23], prior to cryotomy and use in immunohistochemistry and *in situ* hybridization analysis.

Immunohistochemistry

Cryostat sections (6–10 μ m) were freshly cut from biopsies, mounted on 0.1% poly-L-lysine-coated slides and air-dried overnight at 37 °C. Monoclonal antibody staining was detected by the alkaline phosphatase anti-alkaline phosphatase method, as previously described [23]. Normal human serum (NHS, 20%) was used to prevent non-specific binding of the second- and third-layer antibodies. A mouse IgG1 myeloma protein was used as a negative control. The monoclonal antibodies used were CD3, CD4, CD8, CD25, anti-neutrophil elastase and human mast cell tryptase (Dako Cytomation, Cambridge, UK). Polyclonal rabbit anti-mouse Ig and APAAP reagents were also purchased from Dako Cytomation.

For CD4 IFN- γ double-staining slides were pre-treated with 0.1% saponin/phosphate-buffered saline (PBS) for 30 min and incubated overnight with goat anti-human IFN- γ (R & D Systems, Abingdon, Oxford, UK). Biotin anti-goat Ig in saponin/PBS was added for 30 min followed by a Vector APAAP kit (Vector Laboratories Ltd., Peterborough, UK) for a further 30 min. The reaction was developed using Fast Red substrate and was monitored under the microscope. Endogenous peroxidase was blocked for 45 min, washed with PBS prior to staining for CD4 using a vector anti-mouse peroxidase kit and Vector diaminobenzidine (DAB)+nickel substrate. For both single and double enzymatic ICC, slides were encoded and counted in a blinded fashion using an

Olympus microscope (Olympus, London, UK). The number of positive cells was counted in the whole section with an eyepiece graticule at a magnification of $\times 200$ and expressed as the number of positive cells per mm^2 .

Double staining for CD25 and CD4 was performed by fluorescent microscopy as previously described [24]. Briefly, sections were incubated with mouse anti-human CD25 (Dako) overnight at room temperature followed by fluorescein isothiocyanate (FITC)-conjugated, rabbit anti-mouse Ig. Sections were then incubated with phycoerythrin (PE)-conjugated, mouse anti-human CD4 and mounted with fluorescent mounting medium (Dako). The slides were analysed with a Leica TCS SP confocal laser scanning microscope (Leica, Heidelberg, Germany) with a krypton laser. For detection of FITC and PE, the 488 and 568 nm wavelengths were analysed. It was confirmed that there was no fluorescein overlap between the green and red fluorescence channels. The fluorescence of each channel was recorded separately and an extended depth-of-focus image of all the fluorescence within the slides was taken. Subsequently, the images from the two channels were digitally superimposed in pseudocolours to obtain all the information in one picture. Omission or substitution of the primary antibody with a mouse IgG1 was used as a negative control. Positive cells were expressed as the number per square millimetre.

For double staining for CTLA-4 (CD152)/CD4 sections were incubated with Biotin Block reagent (Vector Laboratories Ltd) for 15 min. Following washing, sections were incubated with biotinylated mouse anti-human CTLA-4 in 10% NHS/0.1% saponin/PBS overnight. After washing, the slides were incubated with Streptavidin-DTAF (Jackson ImmunoResearch through Stratech Scientific, Luton, Beds, UK) in 5% NHS/PBS, prior to incubation with CD4-PE (BD Biosciences, Oxford, UK). Sections were mounted with fluorescent mounting medium (Dako). Slides were analysed with a Leica TCS SP confocal laser-scanning microscope and counted as above.

In situ hybridization

This was performed using ^{35}S -labelled antisense riboprobe for IL-5, IL-4, IFN- γ , TGF- β and IL-10, described in detail elsewhere [25, 26]. The number of positive cells was counted as above. For double staining for CD4 immunohistochemistry (IHC) and IL-10 mRNA *in situ* hybridization (ISH), the methods used and the counting were as above with CD4 staining prior to IL-10 mRNA ISH staining.

Statistical analysis

All paired between- and within-subject comparisons were analysed using a statistical package (Minitab release 7; Minitab Inc., State College, PA, USA) with the Wilcoxon signed rank test. A P -value of 0.05 or less was considered as significant.

Results

Adverse events

The treatment was in general well tolerated. However, one patient experienced sneezing and cramping abdominal pains a few minutes after the third dose of Fel d 1-derived peptides.

There were no signs of respiratory or haemodynamic distress. The patient was treated by intramuscular injection of adrenaline. After the adrenaline injection, the patient vomited and felt better. A blood sample, drawn 40 min after treatment with peptides, showed a tryptase level of 9.1 ng/mL (normal range 2–14). The subject was removed from the study and consequently is not included in the results. The absence of respiratory and haemodynamic distress and normal tryptase concentrations indicated that appreciable mast cell degranulation typically associated with anaphylactic reactions had not occurred. A second subject experienced an upper respiratory tract infection immediately prior to the second histamine PC_{20} measurement and was not included in that analysis.

Non-specific bronchial hyper-reactivity and the cutaneous late-phase reaction

Histamine PC_{20} was performed before and 4 weeks after the treatment period. A significantly ($P = 0.02$) greater concentration of histamine was required to induce a 20% reduction in FEV_1 following peptide treatment (Fig. 1a).

Intradermal injection of whole cat dander extract was also performed prior to and 4 weeks after treatment with peptides. A significant reduction ($P = 0.03$) in the magnitude (area in mm^2) of the late-phase reaction was observed at the post-treatment time-point (Fig. 1b). No change was observed in the early-phase reaction at this follow-up point.

Cell infiltration into skin biopsies

The changes in cell numbers at skin challenge sites (diluent vs. cat allergen) were expressed as both 'within-group' and

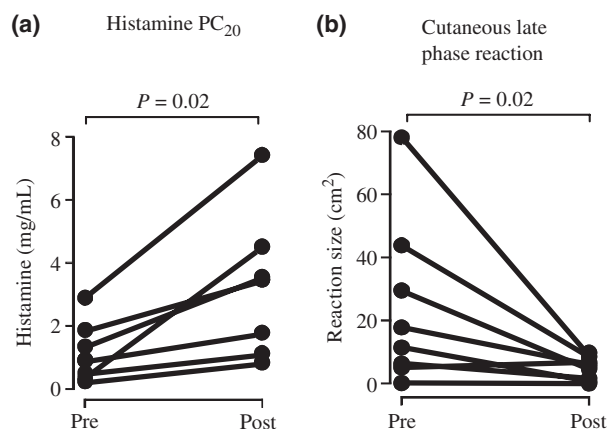


Fig. 1. Effect of T cell peptides on airway hyper-responsiveness and the late-phase skin reaction. Incremental doses of histamine acid phosphatase were inhaled using a breath-activated dosimeter in order to determine the provocative concentration of histamine inducing a 20% decrease (PC_{20}) in baseline forced expiratory volume in 1 s (FEV_1). Measurements were performed at baseline and following a cumulative dose of 45 μg of each of 11 peptides derived from the sequence of Fel d 1. After peptide therapy, higher concentrations of histamine were required in order to induce a 20% reduction in FEV_1 . (b) Subjects were injected intradermally in the volar aspect of one forearm with 10 biological units of whole cat dander allergen extract (ALK-Abelló, Copenhagen, Denmark) or diluent in the other arm. The size of the ensuing cutaneous late-phase reaction was recorded at 24 h post-challenge. Measurements were performed at baseline and repeated 2–8 weeks after completion of peptide therapy. Treatment resulted in a statistically significant reduction in the magnitude of the cutaneous late-phase reaction.

Table 1. Cutaneous cellular infiltration and cytokine mRNA before and after Fel d 1 peptide treatment

	Pre-			Post-			Δ Pre- vs. post- P	Cat allergen (pre-) vs. cat allergen (post-)
	Diluent	Cat	P	Diluent	Cat	P		
Eosinophils	0 (0–1.5)	12.5 (0.7–50.3)	0.01	0 (0–6.3)	37.5 (1.8–203)	0.01	NS	NS
Neutrophils	0.45 (0–19.2)	25.5 (9.8–101)	0.01	1.15 (0–9.4)	80 (29.1–147.0)	0.01	NS	NS
Mast cells	12.9 (6.5–18.5)	15 (1.6–26.6)	NS	8.4 (2.7–27.0)	15.0 (11.3–24.2)	NS	NS	NS
CD3	9.1 (4.2–20.0)	25.8 (14.3–50.3)	0.02	10.5 (5.7–22.3)	39.5 (11.0–63.7)	0.01	NS	NS
CD4	5.5 (0–24.9)	18.9 (0–39.2)	NS	3.7 (1.1–11.2)	18.8 (0.8–39.2)	0.01	NS	NS
CD8	5.3 (0.5–13.6)	13.0 (1.8–29.0)	NS	5.0 (0.5–9.4)	9.3 (1.3–22.4)	0.03	NS	NS
CD25	3.9 (1.0–12.3)	6.7 (1.6–13.1)	NS	3.3 (0.9–7.1)	11.9 (5.2–65.9)	0.02	0.04	0.03
IL-4	0.3 (0–2.4)	12.4 (1.8–19.3)	0.01	0.15 (0–1.6)	10.8 (2.4–17.1)	0.01	NS	NS
IL-5	0.7 (0–1.1)	5.2 (2.9–12.5)	0.01	0.5 (0–1.6)	5.9 (2–10.5)	0.01	NS	NS
IFN- γ	0 (0–1.6)	1.3 (0–5.6)	NS	0 (0–0.8)	1.5 (0–6.1)	0.02	NS	NS
IL-12	0 (0–1.2)	5.1 (0.7–19.6)	0.01	0.5 (0–1.6)	5.7 (0.6–32.1)	NS	NS	NS
IL-10	0 (0)	1.2 (0.3–4.4)	0.01	0.2 (0–0.5)	2.5 (0.7–5.1)	0.01	NS	NS
TGF- β	2.4 (0.9–4.9)	9.4 (1.2–23.8)	0.02	1.7 (0.9–3.3)	12.8 (6.3–40.0)	0.01	NS	0.04

Eight volunteers had two skin biopsies taken 24 h after diluent and whole cat allergen (10 biological units, ALK) challenge, before (pre-) and after (post-) Fel d 1 peptides. The data are shown as the cell count median and (range) for each phenotypic cell marker (immunohistochemistry) and for each mRNA-positive cell for cytokines (*in situ* hybridization). Cell counts at allergen sites are compared with counts at diluent sites using the Wilcoxon signed rank test (P). Significance $P \leq 0.05$. Pre-cell counts at allergen sites are compared with post-cell counts at allergen sites (Δ pre vs. post). The cat allergen pre- vs. the cat allergen post-values were also calculated as between-group differences.

TGF- β , transforming growth factor- β ; NS, non-significant.

'between-group' data. Since there was a small group of subjects, the between-group data were further expressed either as the Δ change (pre-treatment vs. post-treatment, diluent and cat allergen) or simply as the cat allergen pre-treatment vs. the cat allergen post-treatment values as previously described [29]. As shown in Table 1, peptide immunotherapy did not result in a significant change in the recruitment of eosinophils, neutrophils, mast cells or CD3⁺ cells. However, for eosinophils, neutrophils and CD3⁺ cells, both median values and range were greater after therapy. Although there were no significant between-group differences, there were significant within-group increases in allergen-induced CD4⁺ and CD8⁺ cells in the post-treatment biopsies ($P = 0.01$ and 0.03 , respectively). Allergen challenge was associated with elevated numbers of cells expressing CD25 in the post-therapy, but not the pre-therapy biopsies (Table 1). Furthermore, the between-group increase in CD25⁺ cells after peptides was significantly greater than before therapy (Δ change $P = 0.03$; cat dander pre- vs. post- $P = 0.04$). Double staining showed that the numbers of CD4⁺CD25⁺ cells were significantly elevated post- but not pre-peptide treatment ($P = 0.02$; Fig. 2a). Furthermore, the Δ change was significant ($P = 0.02$; Fig. 2a) and there were significantly more CD4⁺CD25⁺ cells at the cat dander challenged sites (pre-treatment vs. post-treatment analysis) ($P = 0.04$; Fig. 2b). Examples of double immunofluorescence staining for CD4 and CD25 are shown in Fig. 4a.

T cell peptide therapy did not alter allergen-induced increases in Th2 cytokine gene expression (IL-4 and IL-5 mRNA⁺ cells) (Table 1). Following peptide treatment, allergen challenge led to local skin recruitment of cells expressing IFN- γ mRNA ($P = 0.02$; Table 1). Double staining for CD4⁺ cells and IFN- γ protein⁺ cells (IHC) revealed a significant increase in the allergen-induced recruitment of IFN- γ ⁺ cells co-localized to CD4⁺ cells following peptide therapy (Figs 3 and 4; Table 2). This was significant when expressed as both the Δ change ($P = 0.03$) as well as

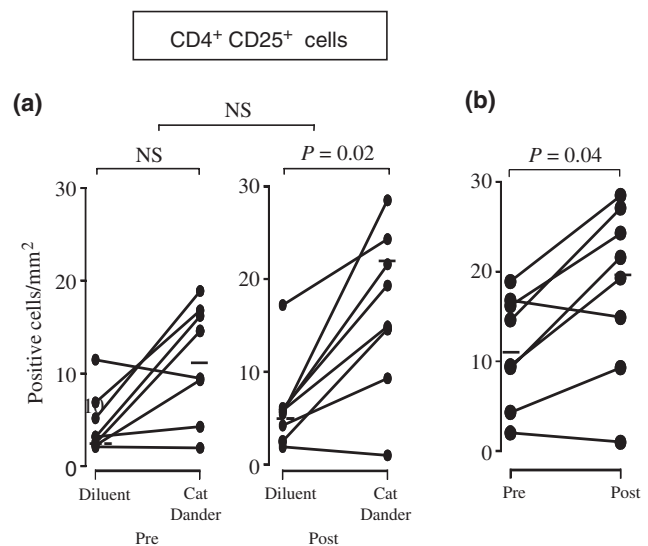


Fig. 2. Effect of T cell peptides on the recruitment of CD4⁺CD25⁺ cells to sites of allergen skin challenge. Punch biopsies were taken from the sites of allergen or diluent challenge after 24 h. Biopsies were sectioned and used to quantify numbers of infiltrating CD4⁺CD25⁺ cells both before and after peptide therapy. (a) Cutaneous challenge with whole cat dander allergen extract results in the recruitment of CD4⁺CD25⁺ cells (between-group Δ change). (b) Comparison of numbers of CD4⁺CD25⁺ cells at sites of allergen challenge before and after peptide therapy (between-group pre-allergen vs. post-allergen).

the pre-treatment vs. post-treatment cat dander-treated sites ($P = 0.03$, Fig. 3b).

The numbers of cells positive for TGF- β 1 mRNA increased after allergen challenge in both pre-treatment ($P = 0.02$) and post-treatment (0.01) groups (Table 1). Although there were significantly more TGF- β 1 mRNA⁺ positive cells following peptide therapy when expressed as a Δ change ($P = 0.04$), double ISH/ICC showed that most of these cells were eosinophils with negligible numbers of CD4⁺/TGF- β ⁺ mRNA cells (data not shown).

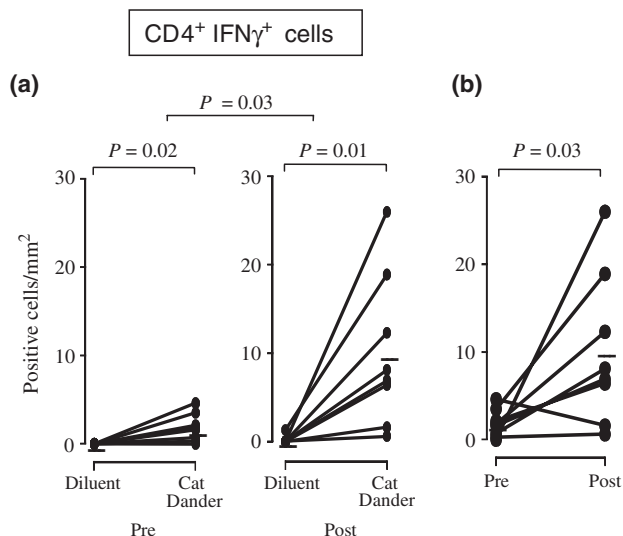


Fig. 3. Effect of T cell peptides on recruitment of CD4⁺IFN-γ⁺ cells to sites of allergen skin challenge. Punch biopsies were taken from the sites of allergen or diluent challenge after 24 h. Biopsies were sectioned and used to quantify numbers of infiltrating CD4⁺IFN-γ⁺ cells both before and after peptide therapy. (a) Cutaneous challenge with whole cat dander allergen extract resulted in the recruitment of CD4⁺IFN-γ⁺ cells (between-group Δ change). (b) Comparison of numbers of CD4⁺IFN-γ⁺ cells at sites of allergen challenge before and after peptide therapy (between-group pre-allergen vs. post-allergen).

Peptide therapy had no significant effect on the whole cat allergen-induced increases in the numbers of cells expressing IL-10 mRNA as measured by ISH (Table 1). Double staining for CD4⁺ cells (IHC) and IL-10 mRNA⁺ cells (ISH) showed no significant changes in the numbers of CD4⁺IL-10⁺ cells either within or between groups (Table 2).

Double staining for CD4⁺CTLA-4⁺ cells (by immunofluorescence) also revealed no effect of peptide administration on CD4⁺CTLA-4⁺ T cell numbers within or between groups (Table 2).

Discussion

The purpose of this study was to determine whether T cell peptides had a similar effect on Th cell recruitment to allergen-challenged tissue sites to that previously reported for conventional SIT [1]. We have already performed a double-blind, placebo-controlled study in which we demonstrated that the peptides inhibited the early- and late-phase skin reactions to whole cat extract [19]. For this reason we did not feel it necessary to have a control (placebo-treated) group for the present design, especially as all the tissue sections were counted in a blind fashion. The peptides had a biological effect as shown by two objective parameters, i.e. a reduction in non-specific bronchial hyper-reactivity (an indirect index of airway inflammation) and a decrease in the whole allergen-induced late-phase skin reaction. However, since these two observations were performed in an 'open' fashion we cannot totally exclude some observer bias.

In our original observation we showed that skin biopsies of allergen-injected sites, taken before and after allergen-SIT, were characterized by an apparent shift in Th2 to Th1 cytokine balance together with an increase in the numbers

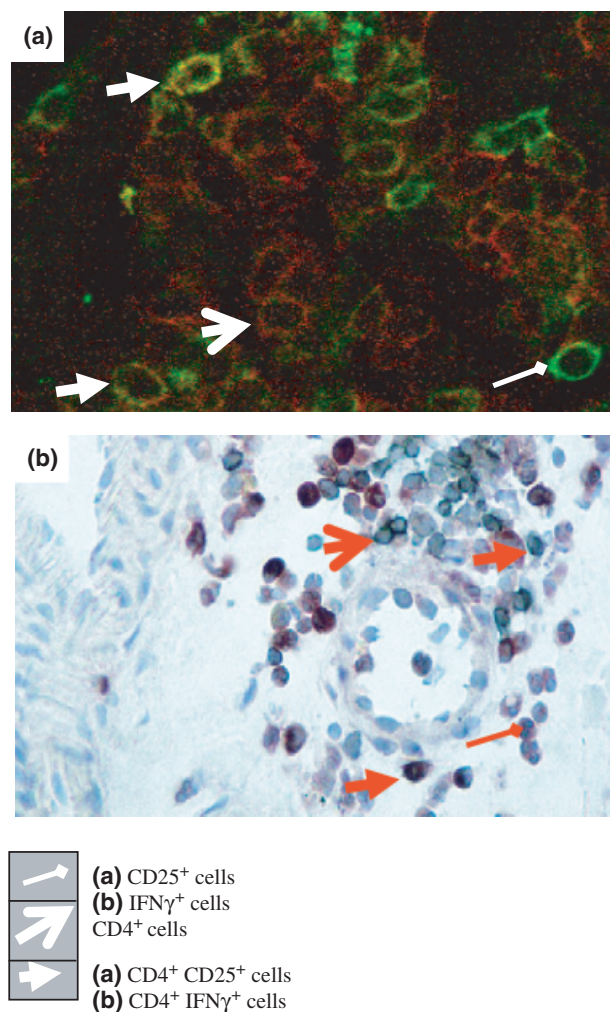


Fig. 4. Labelling of CD4⁺ T cells in allergen-induced late-phase skin reactions. Tissue sections (6–10 μ m) were prepared from punch biopsies (24 h) obtained from cutaneous allergen challenge sites following peptide immunotherapy. (a) Double immunofluorescence labelling of cells expressing CD4 and/or CD25. Single (CD4⁺ or CD25⁺) and double-stained (CD4⁺CD25⁺) cells are indicated. (b) Double immunohistochemical labelling of cells expressing CD4 and/or IFN-γ. Single positive CD4⁺ T cells, single positive IFN-γ cells (predominantly eosinophils) and double-stained CD4⁺IFN-γ⁺ T cells are indicated.

of CD25⁺ T cells [1]. At the time, this observation was interpreted as being indicative of the activation of Th1 cells and since that time, a major focus in developing novel forms of immunotherapy has been to induce allergen-specific Th1 responses.

However, it is now appreciated that the CD4⁺CD25⁺ phenotype is also characteristic of T regulatory cells. The mechanisms through which CD4⁺CD25⁺ T cells and other cell types exert their immunoregulatory effects remain incompletely defined, but have been associated in some models with cell–cell contact [6, 27, 28] and in others with the release of regulatory cytokines such as IL-10 or TGF- β [4, 5, 7].

Recently, Francis et al. [29] have shown that grass pollen immunotherapy results in a population of circulating T cells that express the IL-10⁺CD4⁺CD25⁺ phenotype in response to allergen restimulation. Jutel and colleagues [30] demonstrated decreased production of Th1 and Th2 pro-inflamma-

Table 2. Expression of activation markers and cytokines in CD4 cells in biopsies from allergen challenge sites before and after Fel d 1 peptide treatment

	Pre-			Post-			Δ Pre-vs. post- <i>P</i>	Cat dander (pre-) vs. cat dander (post-)
	Diluent	Cat	<i>P</i>	Diluent	Cat	<i>P</i>		
CD4 CD25	3.2 (2.1–11.5)	9.4 (2–18.90)	NS	5.8 (1.9–17.2)	20.5 (1–28.5)	0.02	0.04	NS
CD4 IFN- γ	0 (0)	1.7 (0–4.6)	0.02	0.16 (0–1.3)	10.1 (0.6–26)	0.01	0.03	0.03
CD4 IL-10	0 (0)	0.35 (0–1.2)	NS	0 (0)	0.55 (0–1.6)	NS	NS	NS
CD4 CTLA-4	0 (0–2.2)	0.7 (0–2.7)	NS	0.4 (0–2.8)	1.7 (0.5–17)	NS	NS	NS

The data are shown are the cell count median and range. The analysis was performed as shown in Table 1.

NS, non-significant.

tory cytokines combined with increased IL-10 and TGF- β following SIT for house dust mite or birch pollen sensitivity. Increased production of IL-10 has also been associated with favourable clinical responses to SIT for bee venom [12] and wasp venom sensitization [14]. Thus, taken together, the presence of increased numbers of CD4⁺CD25⁺ T cells and increased expression of IL-10 may imply a role for regulatory T cells in addition to Th1 cells in successful whole allergen immunotherapy.

In a previous clinical study of peptide immunotherapy, we observed increased production of IL-10 by PBMC, accompanied by decreased production of Th1 and Th2 cytokines [19]. Furthermore, results from the present study indicate that peptide therapy also induced a local population of CD4⁺CD25⁺ cells. The two observations support the concept that peptide immunotherapy may induce IL-10-producing regulatory T cells. However, we observed no increase in mRNA for IL-10 in allergen-challenged skin. In fact, cutaneous challenge was associated with the recruitment of CD4⁺/IFN- γ ⁺ T cells. No change was observed in the total number of IL-10 mRNA⁺ cells or in the numbers of cells expressing other putative markers of regulatory T cell function such as TGF- β and CTLA-4 (Tables 1 and 2).

There were allergen-induced increases in the number of cells expressing mRNA encoding TGF- β ₁ in the pre- and post-peptide-treated skin biopsies (Table 1). However, the between-group analysis did not achieve statistical significance. Furthermore, no changes were seen in overall CTLA-4 expression in biopsy sections before and after therapy, or in CD4⁺/CTLA-4⁺ cells (Table 2). A role for enhanced levels of surface expression of CTLA-4 on T cells cannot be excluded since the staining techniques used did not distinguish between surface and intracellular protein expression.

Our previous report of elevated levels of IL-10 production from 6th day PBMC cultures in response to allergen challenge *in vitro* following peptide therapy was not reproduced in the skin. The reason for this may be that IL-10 production in allergen/PBMC cultures appears to be primarily monocyte-derived (unpublished observations) at the 24 h time point (time of skin biopsy). A recent study of whole allergen immunotherapy demonstrated significant increases in IL-10 expressing cells at mucosal sites of natural allergen challenge [15]. A potentially important effect of peptide immunotherapy may be to indirectly modulate IL-10 production by monocytes and this is currently under investigation. Similarly, IFN- γ production in response to allergen in the skin was increased in contrast to decreases in production of the same cytokine by PBMCs in earlier studies [18, 19]. Thus, the discrepancies in

these findings may reflect differences in the physical location of tissue sampling, the cellular source of cytokine production and the fact that PBMC were not specifically localized, by allergen challenge *in vivo*, prior to sampling.

We also need to attempt to reconcile our observations with an earlier report in which we showed that IL-10⁺ cells increased in cutaneous biopsies of patients undergoing wasp venom immunotherapy [14]. Either there are fundamental differences between peptide therapy and SIT, or the profile of infiltrating Th cells may be related to the intensity of therapy. Thus, the subjects in the present study had only 45 μ g of peptide mixture over the study period. Higher dosing over longer periods of time may induce Th cells with a more regulatory cell profile. However, the regimen used in this study was sufficient to induce activated Th1-like cells and to have clinical effects, i.e. changes in AHR and inhibition of the late-phase reaction.

In summary, skin biopsies taken from sites of allergen challenge before and after therapy demonstrate that peptide treatment is associated with immunomodulation characterized by recruitment of increased numbers of CD4⁺CD25⁺ T cells and increased numbers of CD4⁺ cells producing IFN- γ . In contrast to findings in the blood with previous peptide studies, and the skin in previous SIT studies [14, 18, 19], cutaneous responses to allergen using the current experimental design were not associated with elevated expression of the regulatory cytokine IL-10 following peptide treatment.

The results described in this study are compatible with a role for enhanced Th1 responses in the skin following successful peptide immunotherapy. Previous findings in *ex vivo* cultures of blood cells are consistent with IL-10-dependent regulation, but the majority of the IL-10 may be monocyte-, rather than T cell-derived. The findings are consistent with observations in whole allergen immunotherapy and may help to elucidate the mechanisms underlying effective immunomodulation.

Acknowledgements

The authors wish to thank Dr D. S. Robinson for critical review of the manuscript, Julia Barkans for technical assistance and Karen Shirley for assistance in monitoring the study volunteers. C. A. is the recipient of the TV James Fellowship of the British Medical Association. M. L. is an Asthma UK Senior Research Fellow. This study was funded through grants from the British Medical Association, Asthma UK and Medical Research Council (UK).

References

- 1 Varney VA, Hamid QA, Gaga M et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest* 1993; 92:644–51.
- 2 Hamid QA, Schotman E, Jacobson MR, Walker SM, Durham SR. Increases in IL-12 messenger RNA+ cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy. *J Allergy Clin Immunol* 1997; 99:254–60.
- 3 Bacchetta R, Bigler M, Touraine JL et al. High levels of interleukin 10 production *in vivo* are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* 1994; 179:493–502.
- 4 Groux H, O'Garra A, Bigler M et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; 389:737–42.
- 5 Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* 1996; 183:2669–74.
- 6 Ng WF, Duggan PJ, Ponchel F et al. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 2001; 98:2736–44.
- 7 Zuany-Amorim C, Sawicka E, Manlius C et al. Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat Med* 2002; 8:625–9.
- 8 Baker KS, Roncarolo MG, Peters C, Bigler M, DeFor T, Blazar BR. High spontaneous IL-10 production in unrelated bone marrow transplant recipients is associated with fewer transplant-related complications and early deaths. *Bone Marrow Transplant* 1999; 23:1123–9.
- 9 Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* 1996; 184:19–29.
- 10 Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001; 2:725–31.
- 11 Hansen G, McIntire JJ, Yeung VP et al. CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J Clin Invest* 2000; 105:61–70.
- 12 Akdis CA, Blaser K. IL-10-induced anergy in peripheral T cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy. *FASEB J* 1999; 13:603–9.
- 13 Stephens LA, Mottet C, Mason D, Powrie F. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity *in vitro*. *Eur J Immunol* 2001; 31:1247–54.
- 14 Nasser SM, Ying S, Meng Q, Kay AB, Ewan PW. Interleukin-10 levels increase in cutaneous biopsies of patients undergoing wasp venom immunotherapy. *Eur J Immunol* 2001; 31:3704–13.
- 15 Nouri-Aria KT, Wachholz PA, Francis JN et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 2004; 172:3252–9.
- 16 Norman PS, Ohman JL, Long AA et al. Treatment of cat allergy with T-cell reactive peptides. *Am J Respir Crit Care Med* 1996; 154:1623–8.
- 17 Muller U, Akdis CA, Fricker M et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998; 101:747–54.
- 18 Oldfield WL, Kay AB, Larche M. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyporesponsiveness in atopic allergic asthmatic subjects. *J Immunol* 2001; 167:1734–9.
- 19 Oldfield WL, Larche M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 2002; 360:47–53.
- 20 Cockcroft DW, Ruffin RE, Frith PA et al. Determinants of allergen-induced asthma: dose of allergen, circulating IgE antibody concentration, and bronchial responsiveness to inhaled histamine. *Am Rev Respir Dis* 1979; 120:1053–8.
- 21 Haselden BM, Kay AB, Larche M. Immunoglobulin E-independent major histocompatibility complex-restricted T cell peptide epitope-induced late asthmatic reactions. *J Exp Med* 1999; 189:1885–94.
- 22 Alexander C, Oldfield WL, Shirley K, Larche M, Kay AB. A dosing protocol of allergen-derived T cell peptide epitopes for the treatment of allergic disease. *J Allergy Clin Immunol* 2001; 107:716 abs.
- 23 Frew AJ, Kay AB. The relationship between infiltrating CD4+ lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* 1988; 141:4158–64.
- 24 Hamid Q, Barkans J, Robinson DS, Durham SR, Kay AB. Co-expression of CD25 and CD3 in atopic allergy and asthma. *Immunology* 1992; 75:659–63.
- 25 Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol* 1993; 8:35–42.
- 26 Ying S, Humbert M, Barkans J et al. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 1997; 158:3539–44.
- 27 Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; 193:1285–94.
- 28 Hoyne GF, Le RI, Corsin-Jimenez M et al. Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells. *Int Immunol* 2000; 12:177–85.
- 29 Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003; 111:1255–61.
- 30 Jutel M, Akdis M, Budak F et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003; 33:1205–14.