

Oligoclonal Analysis of the Atopic T Cell Response to the Group 1 Allergen of *Cynodon dactylon* (Bermuda Grass) Pollen: Pre- and Post-Allergen-Specific Immunotherapy

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Key Words

Bermuda grass pollen · Cyn d 1 · Allergy · Immunotherapy · T cell epitopes · Cytokines

Abstract

Background: Bermuda grass pollen (BGP) is an increasingly important seasonal aeroallergen in Australia and other subtropical and temperate regions. BGP shares minimal allergenic cross-reactivity with pollens of rye grass or other Pooideae grasses often used for desensitization regimens in grass pollen allergy. Current allergen immunotherapy is seldom used in asthmatic patients due to IgE-mediated side effects. Since clinically effective immunotherapy is linked with altered allergen-specific T cell response, characterisation of human T cell reactivity to Cyn d 1, the major B cell allergen of BGP, should permit the design of effective and safe immunotherapy for BGP allergy. **Methods:** Short-term BGP-specific CD4⁺ T cell lines were established from peripheral blood of 14 BGP-sensitive patients before and after conventional 50% BGP and 50% 7-grass mix subcutaneous specific allergen immunotherapy (SIT). T cell diversity of antigen specificity and function was assessed by proliferation

and cytokine production to BGP, Cyn d 1 and Cyn d 1 peptides. **Results:** Three highly immunogenic regions of Cyn d 1 were identified in 13/14 patients pre-SIT: Cyn d 1 (109–128), (181–209) and (217–241). The SIT regimen was clinically efficacious. Following SIT, decreased proliferation to BGP, Cyn d 1 and Cyn d 1 peptides was observed with a marked decrease in the IL-5:IFN- γ ratio. **Conclusions:** Cyn d 1 is a major T cell allergen of BGP. Decreased Cyn d 1-specific IL-5 dominant T cell responses were observed in association with clinically effective treatment with the 50% BGP and 50% 7-grass mix. Identified dominant T cell regions of Cyn d 1 should facilitate safer vaccine development for BGP-induced asthma in addition to rhinitis.

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Introduction

Bermuda or couch grass (*Cynodon dactylon*) pollen (BGP) is a clinically important seasonal aeroallergen in subtropical climates worldwide and in temperate climates where Bermuda grass is increasingly used in lawn mixes and to prevent soil erosion. In Australia, rye grass pollen

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is generally regarded as the most important seasonal allergen source, but of 736 patients in our allergy data base with seasonal asthma and/or rhinitis, 627 (85%) are sensitised to both BGP and rye grass pollen and only 100 (14%) are sensitised to rye grass pollen alone [O'Hehir, unpubl. obs.]. Patients who have clinical seasonal allergy due to rye grass have symptoms in spring, but with BGP allergy, peak symptoms extend into late summer in the southern hemisphere.

Pharmacotherapy is the mainstay of treatment of allergic disease, but for appropriately chosen subjects, allergen specific immunotherapy (SIT) offers the opportunity to modify the natural course of the disease and has been shown to be highly efficacious and long-lasting in grass pollen allergy [1]. Nevertheless, although SIT is accepted clinical practice for treatment of seasonal rhinitis, the treatment is seldom used in asthmatic patients for fear of inducing severe asthma or even anaphylaxis. In our allergy clinic, 50% of the patients with Bermuda grass allergy experience asthma and rhinitis. Therefore, there is a demand for safer SIT regimens to permit wider application for treatment of BGP sensitivity. With a growing appreciation of the critical role of T cells in the elicitation and regulation of the immune response to allergens, new T-cell-targeted strategies for SIT are being explored. Preparations which lack IgE-binding reactivity but contain dominant T cell epitopes should be safe and effective [2, 3].

Previous studies have identified multiple IgE-reactive proteins of BGP using sera from BGP-allergic patients, with predominant humoral recognition of one protein Cyn d 1 (>76%), thus termed the major allergen of BGP [4–7]. The cDNA encoding Cyn d 1 has been cloned and from the nucleotide sequence, the primary amino acid sequence has been deduced [8]. We previously reported an analysis of human peripheral blood T cell recognition of BGP in atopic BGP-allergic and non-atopic subjects [9]. Both groups showed T-cell-proliferative responses to BGP, but the magnitude of response on average was greater in the atopics. Studies with other allergens indicate that a predominant Th2-type cytokine response to allergens further distinguishes those individuals with an allergic phenotype [10]. Clinical efficacy of SIT is reported to be associated with decreased production of IL-4 and IL-5 by allergen-stimulated T cells [2]. Therefore, a thorough knowledge of allergen-specific T cell responses is required for improved SIT preparations. There are no previous reported systematic studies of T cell reactivity to BGP or Cyn d 1.

In this study, we have performed the first detailed analysis of the repertoire and function of human CD4⁺ T cells

reactive with the major allergen of BGP, Cyn d 1. In addition, to elucidate mechanisms of SIT, we tested BGP-sensitive patients before and after standard immunotherapy using a tailored depot preparation containing 50% BGP and 50% 7-grass mix. This preparation was used since there is minimal cross-reactivity between pollen of grasses of the Pooideae subfamily and BGP [11–14]. Therefore, the standard 7-grass mix extract (rye, cocksfoot, bent, Kentucky blue, sweet vernal, timothy and meadow fescue), frequently used for immunotherapy of grass pollen allergy, is unlikely to relieve symptoms due to BGP sensitisation. Using oligoclonal T cell blasts, Cyn d 1 was shown to be a major T cell allergen of BGP and three highly immunogenic regions of Cyn d 1 were identified.

Following successful SIT, there was a marked decrease in the allergen specific T-cell-proliferative response accompanied by a decrease in the IL-5:IFN- γ ratio. This study provides pivotal data for the development of effective and safe SIT for BGP allergic disease.

Materials and Methods

Patients and Immunotherapy Protocol

Fourteen patients (8 females and 6 males of mean age 33.4 years) from the Asthma and Allergy Clinic, Alfred Hospital, Melbourne, Australia were studied (table 1). All patients suffered from severe seasonal rhinitis and/or asthma with symptoms extending into late summer corresponding to the peak of the BGP season. BGP-specific IgE was present in all subjects as determined by positive skin prick tests to BGP extract (wheal diameters ≥ 5 mm) as well as positive enzyme allergosorbent test (EAST, Kallestad Allercoat Sanofi-Pasteur Diagnostics; table 1).

Patients were administered subcutaneous SIT with 2-weekly injections of an alum-precipitated depot preparation containing 50% BGP and 50% 7-grass mix (Allpyral[®] specifically prepared and generously provided by Bayer Australia Ltd.) for 20 weeks out of the pollen season, according to the Alfred Hospital standard regimen (cumulative antigen dose 15,570 PNU per year). Heparinized peripheral blood was obtained from all patients during the pollen season before initiation of desensitization (n = 14), following 1 year of treatment (n = 14) and after 2 years of treatment (n = 7). Changes in clinical symptomatology were documented using visual analogue scores completed by the patients (100% equals most severe symptoms and 0% equals no symptoms; fig. 1). and by patient-reported changes in medication usage. Approval was obtained from the Alfred Hospital Ethics Committee with informed written consent from each patient. Daily pollen counts were kindly provided by the School of Botany, University of Melbourne.

MHC Class II Typing

HLA-DRB1, DQB1 and DPB1 allele typing was performed using the standard methods outlined in the 11th International Histocompatibility Workshop [15]. Briefly, exon two polymorphism was determined using a series of biotinylated oligonucleotide probes that were hybridised to PCR-amplified DNA immobilised on Nylon mem-

Fig. 1. Visual analogue scores of patients pre- and post-SIT. a Patients treated with two courses of SIT. b Patients treated with one course of SIT.

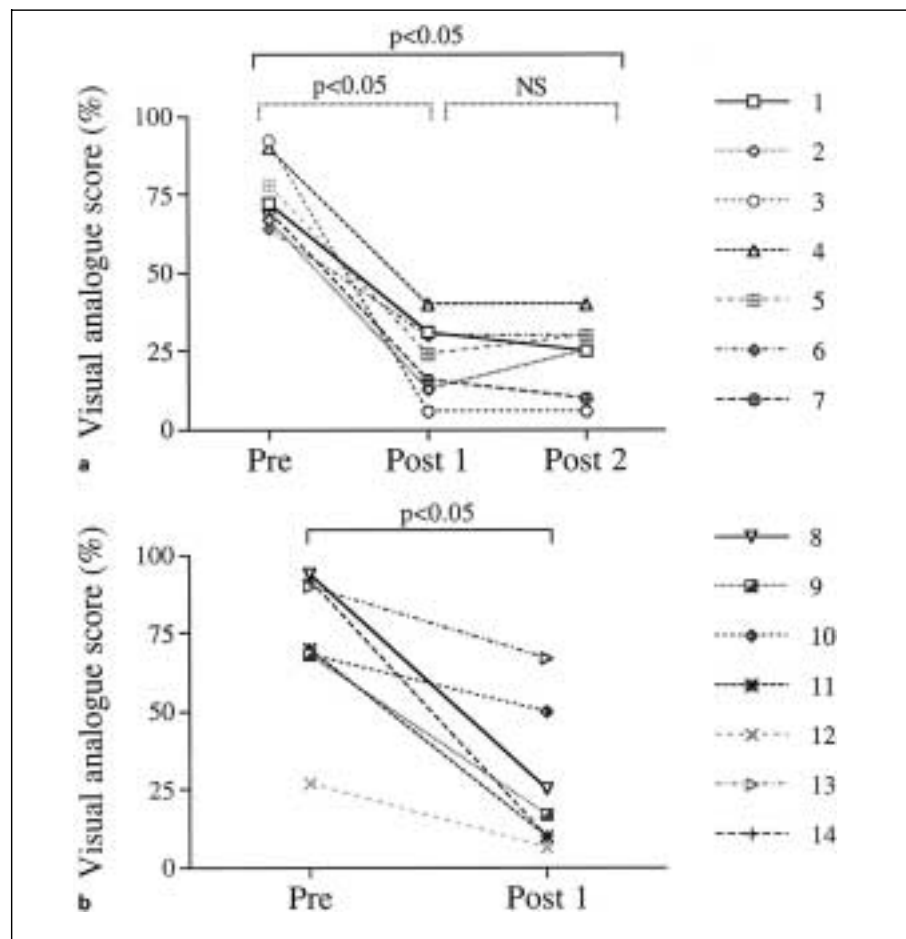


Table 1. Clinical characteristics of the BGP-allergic patients and BGP-specific IgE before and after 1 and 2 (patients 1–7) courses of SIT

Patient No.	Sex	Age	Seasonal rhinitis	Asthma	BGP skin test reactivity, mm			BGP EAST score (AEU/ml)		
					pre-SIT	post 1-course SIT	post 2-course SIT	pre-SIT	post 1-course SIT	post 2-course SIT
1	M	22	+++	++	12	10	7	ND	3 (5.33)	3 (14.54)
2	F	47	+++	+	10	8	8	2 (1.86)	3 (10.84)	ND
3	F	33	+++	+	11	11	11	ND	3 (16.08)	3 (16.55)
4	F	36	+++	–	7	4	3	3 (6.44)	3 (5.72)	ND
5	F	24	+++	++	6	5	8	3 (4.84)	3 (11.83)	ND
6	F	45	+++	–	14	11	11	1 (0.45)	2 (0.87)	3 (7.12)
7	F	30	+++	+	ND	ND	8	2 (1.64)	ND	2 (2.40)
8	F	22	+++	++	7	6		2 (2.70)	3 (3.823)	
9	M	22	+++	+	11	5		3 (7.03)	3 (11.05)	
10	M	27	+++	–	ND	13		3 (11.82)	3 (13.68)	
11	M	30	+++	++	5	11		3 (5.15)	4 (>17.5)	
12	F	47	+++	–	7	7		3 (5.29)	3 (9.28)	
13	M	27	+++	++	10	15		3 (7.06)	ND	
14	M	55	+++	–	10	5		0 (<0.18)	2 (0.78)	

ND = Not done; M = male; F = female; AEU = Allercoat EAST units.

(1–20)	AIGDKPGPNITATYGNKWLE
(10–29)	ITATYGNKWLEAKATFYGSN
(19–38)	LEAKATFYGSNPRGAAPDDH
(28–47)	SNPRGAAPDDHGGACGYKDV
(37–56)	DHGGACGYKDVKPPFDGMT
(46–65)	DVDKPPFDGMTACGNEPIFK
(55–74)	MTACGNEPIFKDGLGCGACY
(64–83)	FKDGLGCGACYEIKCKEPVE
(73–92)	CYEIKCKEPVECSGEPVLVK
(82–101)	VECSGEPVLVKITDKNYEHI
(91–110)	VKITDKNYEHIAAYHFDLSG
(100–119)	HIAAYHFDLSGKAFGAMAKK
(109–128)	SGKAFGAMAKKGQEDKLRKA
(118–137)	KKGQEDKLRKAGELTLQFRR
(127–146)	KAGELTLQFRRVKCKYPSGT
(136–155)	RRVKCKYPSGTKITFHIEKG
(145–164)	GTKITFHIEKGSNDHYLALL
(154–173)	KGSNDHYLALLVYAAGDGN
(163–182)	LLVYAAGDGNIVAVDIKPK
(172–191)	GNIVAVDIKPKDSDEFIPMK
(181–200)	PKDSDEFIPMKSSWGAIWRI
(190–209)	MKSSWGAIWRIDPKKPLKGP
(199–218)	RIDPKKPLKGPFSIRLTSEG
(208–227)	GPFSIRLTSEGGAHLVQDDV
(217–236)	EGGAHLVQDDVIPANWKPD
(222–241)	LVQDDVIPANWKPDVYTSK
(227–246)	VIPANWKPDVYTSKLQFGA

Fig. 2. Amino acid sequence of the overlapping peptides of Cyn d 1. Residues are indicated with the single letter code.

branes. Complementary probes were detected using a streptavidin-alkaline phosphatase conjugate and the chemiluminescent substrate CDP-Star (Roche Diagnostics Pty Ltd). DPB1 hybridisation was preceded by a sequence-specific priming to divide DPB1 alleles into six groups based on polymorphism at amino acid positions 8–9 and 84–85 [16]. For each locus, new probes and primers were added to cover sequence polymorphism not described in the original protocol.

Antigens

Bermuda Grass Pollen. BGP was purchased from Greer Laboratories Inc. (Lenoir, N.C., USA) as dry, non-defatted pollen. One gram of pollen was extracted in 5 ml of 1 mM NH_4HCO_3 overnight at 4°C on a rotating wheel. After centrifugation, the supernatant was dialyzed against PBS overnight. The extract was filtered through a 0.2-µm filter. The protein content of the extract was determined using the Bio-Rad Microassay (Bio-Rad, USA).

Cyn d 1. Cyn d 1 was purified from the BGP extract first undergoing an 80% ammonium sulphate precipitation. The supernatant from the precipitation was then passed through a G-75 Sephadex gel column (Pharmacia, Sweden). The fractions containing Cyn d 1 were pooled following analysis on SDS-PAGE and immunoblotting using a Cyn d 1-specific monoclonal antibody (3A2) [17]. The protein content was determined using the Bio-Rad Microassay.

Peptides. Synthetic peptides (20-mers overlapping by 11 or, for the 2 N-terminal peptides, 15 residues) spanning the entire length of

the Cyn d 1 molecule were purchased from Mimotopes (Clayton, Australia; fig. 2). Peptide sequences were based on the Cyn d 1 CD1 isoform [8].

Mitogenicity and Toxicity. Mitogenicity of BGP, Cyn d 1 and Cyn d 1 peptides was excluded by culturing with oligoclonal house dust mite-reactive CD4+ T cells in the presence of irradiated peripheral blood mononuclear cells (PBMC), and toxicity was excluded by co-culture of antigens with the house dust mite-reactive T cells in the presence of IL-2 (data not shown).

Generation of BGP-Specific T Cell Lines

BGP-specific T cell lines were isolated with the use of our well-established methods for the generation of allergen-specific T cell populations [18, 19]. Briefly, PBMC were separated from heparinized venous blood by density gradient centrifugation over Ficoll Paque (Pharmacia, Sweden). PBMC were initially stimulated for 1 week in 24-well plates (Costar, USA; 2.5×10^6 PBMC/well) with a previously determined optimal concentration of BGP extract (50 µg/ml) for 7 days in complete medium (RPMI-1640 medium, Gibco Life Technologies, supplemented with 2 mmol/l *L*-glutamine, 100 IU/ml penicillin/streptomycin, and 5% screened, heat-inactivated human AB+ serum, Sigma Chemical Company, USA) at 37°C, 5% CO_2 . A 2-week line was established by restimulating the T cell blasts with BGP extract in the presence of irradiated (3,000 rad) autologous PBMC every 7 days, with the addition of interleukin-2 (5% vol/vol; Lym-

phocult-TLF, Biotest Folex, Frankfurt, Germany) every 3–4 days. In all experiments, T cells were rested for 7 days after the last addition of antigen and antigen-presenting cells (APC) before use in proliferation assays. We have previously shown that CD4⁺ T cells are preferentially expanded in these cultures.

Oligoclonal T Cell Proliferation Assays

Oligoclonal T cell blasts (5×10^4 /well) from the BGP-specific T cell lines were stimulated in triplicate with BGP extract (25, 50, 100 µg/ml), purified Cyn d 1 (2.5, 5, 10 µg/ml) and the Cyn d 1 peptides (1, 10 µg/ml) in the presence of an equal number of irradiated autologous PBMC as APC for 72 h in 96-well round bottom plates (ICN Biomedicals, USA). Cultures of T cells and APC in the absence of antigen or with added IL-2 were included as negative and positive controls, respectively. After 72 h, cultures were pulsed with 1 µCi/well of tritiated methyl thymidine (³HTdR; Amersham, USA) and harvested 16 h later onto glass fibre filters. Proliferation as correlated with ³HTdR incorporation was measured by liquid scintillation spectroscopy. Results are expressed as change in mean counts per minute above background (Δ cpm) or as a stimulation index (SI, cpm of antigen stimulated T cells divided by cpm of unstimulated T cells) to allow easier comparison between subjects with different background incorporation. SI values ≥ 2.5 are considered significant.

Production of IL-5 and IFN- γ by BGP-Specific Oligoclonal T Cells

Production of IL-5 and IFN- γ by cultured oligoclonal T cells was assessed by harvesting supernatants from proliferation assays at 48 h after stimulation, with replacement by an equal volume of complete medium, and measurement of cytokine levels by sandwich ELISA. White 96-well maxisorp ELISA plates (Nalgene NUNC, Denmark) were coated with rat anti-mouse/human IL-5 (PharMingen, USA) or mouse anti-human IFN- γ (Endogen, USA) at 2 µg/ml and incubated overnight at 4°C. The plates were washed with 0.5% Tween 20/PBS and blocked with 1% BSA/PBS for 1 h at room temperature. The plates were washed again and the culture supernatants from pooled triplicate assays added in duplicate to the wells. A standard curve was established using known concentrations of human recombinant IL-5 (PharMingen, USA) and IFN- γ (Endogen, USA). The plates were incubated overnight at 4°C. After washing the plates again, the wells were incubated with either 1 µg/ml biotinylated rat anti-human IL-5 (PharMingen, USA) or 0.5 µg/ml biotinylated mouse anti-human IFN- γ (Endogen, USA) for 1 h at room temperature. After washing the plates, streptavidin-biotinylated horseradish peroxidase (Amersham, USA), at a dilution of 1:2,000, was added to each well and incubated for 45 min at room temperature. The plates were once again washed and 100 µl of the ECL Chemiluminescence reagent (NEN Life Science Products, USA), made up by mixing one part enhanced luminol reagent with one part oxidizing reagent, was added to each well. The plates were read on a LumiCount microplate glow luminometer (Packard Instrument Company, USA). Standard curve construction and determination of cytokine levels were performed using Packard I-Smart software. The lower limit of detection of the IL-5 and IFN- γ assays was 10 pg/ml.

Statistics

Statistical significance of differences between results obtained at various time points of SIT was analysed by a Wilcoxon Signed Rank test. The tests with p values of less than 0.05 were considered significant.

Results

Clinical Efficacy of Immunotherapy

All 14 patients demonstrated a marked improvement in their symptoms as assessed by the visual analogue scores reported by the patients during the pollen season (fig. 1). This was accompanied by decreased medication usage. As anticipated from published studies [20–23], the BGP-specific IgE measurements showed no consistent changes during the time of the study (table 1). Daily seasonal pollen counts were comparable over the three seasons (data not shown).

Oligoclonal T Cell Responses to BGP Allergens

The 2-week BGP-specific T cell lines from all 14 BGP allergic patients showed strong proliferative T cell responses to the crude BGP extract pre-SIT with 13/14 patients also responding to Cyn d 1. A representative proliferation assay expressed as Δ cpm for 1 patient is shown in detail in figure 3a. Following SIT, there was a marked decrease in proliferative responses to BGP and Cyn d 1. Proliferation to BGP following 1 year of SIT was decreased in 12/14 patients (fig. 4a, b). Patient 5 showed a slight decrease in proliferation after 1 year of SIT but, interestingly, T cell reactivity to BGP was increased after 2 years. Patient 11 showed a similar increase in T cell recognition of BGP at 1 year. A decrease in proliferation to Cyn d 1 was observed in 11/13 Cyn d 1-reactive patients following 1 year of SIT (fig. 4c, d). After 2 years of SIT, only minimal antigen-specific T cell proliferation was observed. This was not due to induction of cytotoxicity, as at all three time points strong proliferation to IL-2 was observed for all individuals (fig. 3a, data not shown).

Epitope Mapping of Cyn d 1

To identify the highly immunogenic regions of Cyn d 1, proliferative responses of oligoclonal T cells to the synthetic Cyn d 1 peptides were examined pre-SIT. T cell reactivity to one or more Cyn d 1 peptides was identified in all 13 Cyn d 1-reactive patients, but the pattern of T cell recognition differed between individuals. There was no peptide recognition by the Cyn d 1-non-reactive patient. Four peptides [Cyn d 1(10–29), (46–65), (55–74) and (199–218)] failed to elicit proliferation in any subject. Regions of high immunogenicity were detected where peptides elicited proliferation in 6 or more patients: peptides Cyn d 1(109–128) and (217–236) each elicited responses in 8 patients and peptides Cyn d 1(181–200), (190–209) and (222–241) each induced T cell responses in 6 patients (fig. 5a).

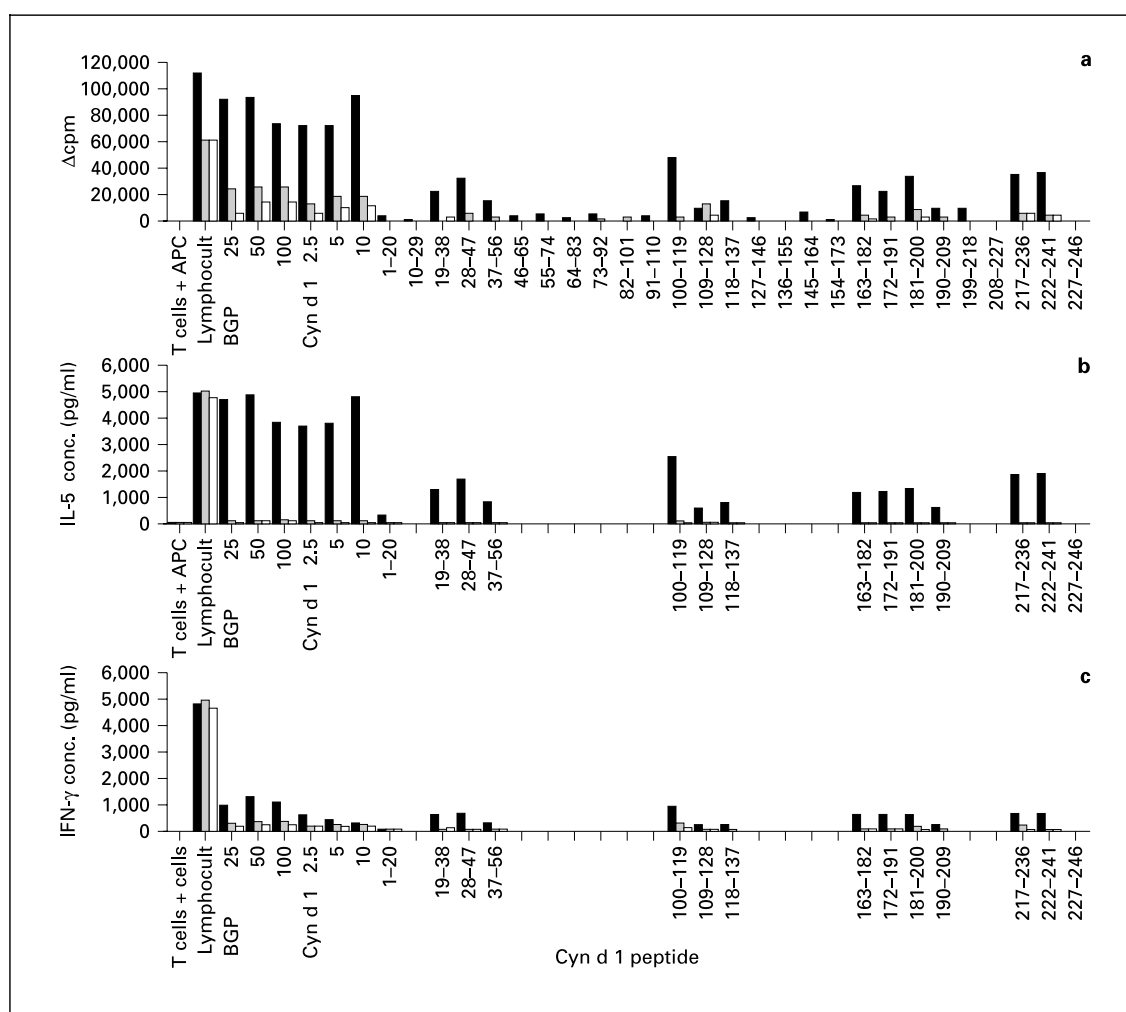


Fig. 3. Representative changes in T cell proliferation (a) and cytokine production (b, c) following SIT (patient 2). Proliferation pre-SIT (■; background = 2,600 cpm), post 1-SIT (one course; ▨; background = 4,700 cpm) and post 2-SIT (two courses; □; background = 8,000 cpm) are shown. Production of IL-5 (b) and IFN-γ (c) in culture supernatants was assessed by ELISA. Cytokine levels were measured for peptides which elicited a proliferative response ≥ 2.5 times background pre-SIT. Values indicate averages of duplicate samples.

HLA DRB1, DQB1 and DPB1 allele frequencies in 13/14 patients were compared to those observed in a healthy control population and were analyzed for association with a response to one or more particular peptide sequences. No unequivocal HLA associations were observed.

As observed with unfractionated BGP extract and Cyn d 1, following SIT, recognition of the Cyn d 1 peptides decreased markedly (fig. 5), with negligible determinant spreading. For the 7 patients who had 2 years of SIT, only 2 peptides elicited a proliferative response, each in 2 patients (fig. 5c).

Cytokine Profile of Reactive T Cells

IL-5 and IFN-γ were secreted by the BGP-specific T cell lines in response to stimulation with BGP, Cyn d 1 and selected Cyn d 1 peptides that caused T cell proliferation (e.g. fig. 3). High levels of both cytokines were produced pre-SIT but post-SIT, there was a marked decrease in IL-5 production in 13/14 patients following their one or two courses of SIT (fig. 3b and data not shown). IFN-γ levels also decreased post-SIT in 10/14 patients, however, to a lesser extent compared to IL-5 (fig. 3c, data not shown). IFN-γ secretion was enhanced in 4 patients (8, 9, 11, 13), each of whom had only undertaken one course of

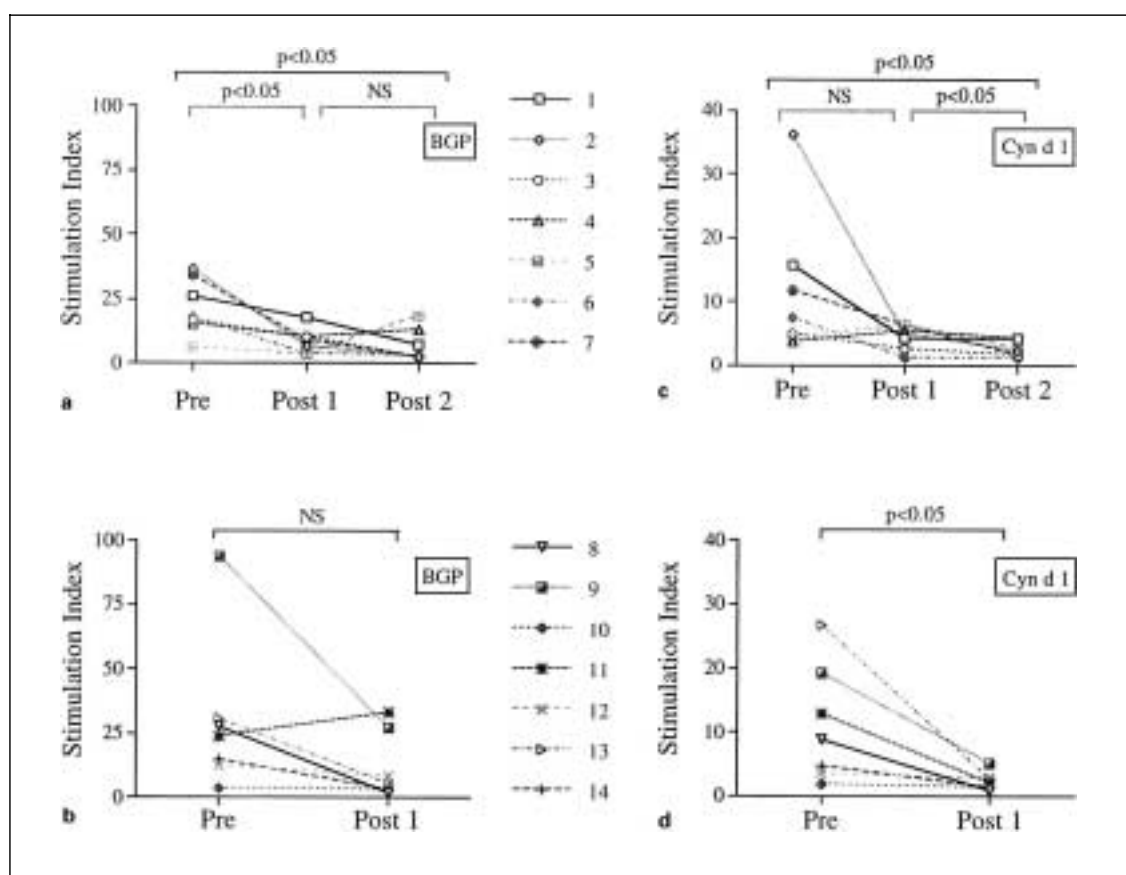


Fig. 4. Changes in T cell proliferative responses to BGP (a, b) and Cyn d 1 (c, d) in BGP-allergic patients. The maximal response for each antigen is expressed as a stimulation index and compared pre-SIT and post-SIT, one course (post 1), two courses (post 2).

SIT when studied. Patient 13 showed increased IL-5 secretion after 1 year of SIT, but IFN- γ secretion was enhanced to a greater degree (fig. 6b, d). After 2 years of SIT, there was negligible secretion of either cytokine by any patient, consistent with the low level of T cell proliferation observed at this time point.

There was a marked shift in the IL-5:IFN- γ ratio during SIT. Using an arbitrary ratio of 1 as a point of comparison, 11/14 patients showed a shift in the IL-5:IFN- γ ratio from ≥ 1 pre-SIT to ≤ 1 at 1 year post-SIT for T cells stimulated with both BGP and Cyn d 1 (fig. 6). Despite the increased T-cell-proliferative response observed after stimulation with BGP following SIT in patients 5 and 11, the IL-5:IFN- γ ratio decreased in both these patients and clinical symptoms also decreased. After 2 years of SIT, the ratio either remained constant at a low level or continued to decline.

The profile of the IL-5:IFN- γ ratio elicited by peptide stimulation mirrored T cell proliferation (data not shown). After 1 year of SIT, only seven peptides induced a ratio of ≥ 1 in at least 1 patient, and after 2 years of SIT, this was virtually abrogated with only one peptide inducing a ratio of > 1 in 2 patients.

Discussion

BGP is emerging as an important seasonal allergen source in subtropical and temperate climates worldwide with 1 major allergen, Cyn d 1, known to elicit predominant IgE-mediated humoral recognition. Although BGP is known to be a predominant cause of seasonal rhinitis and asthma, with sensitised patients suffering pollen-triggered symptoms through until late summer, BGP is not adequately covered in allergen immunotherapy preparations

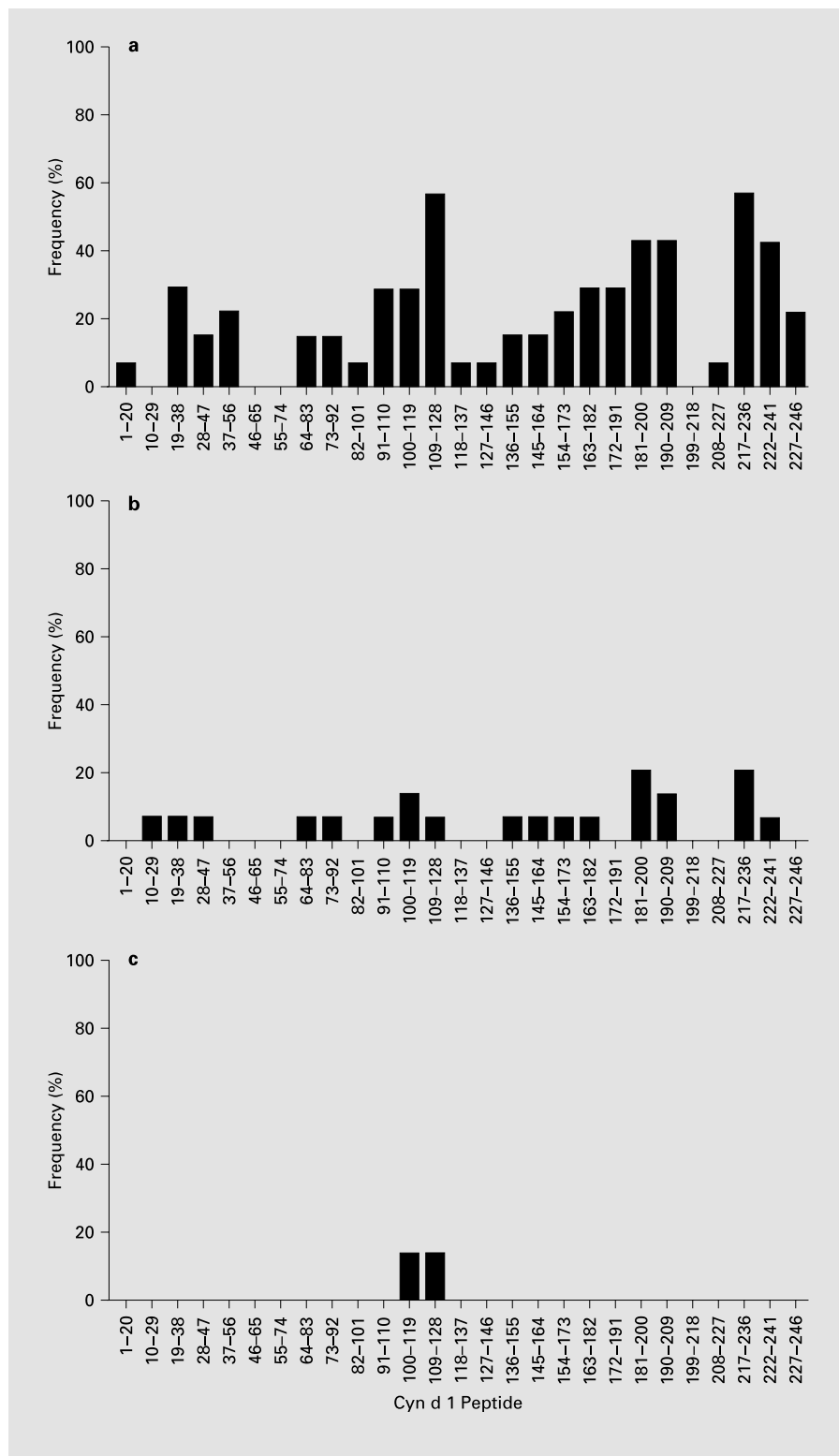


Fig. 5. Change in frequency of recognition of peptides ($SI \geq 2.5$) following SIT in BGP-allergic patients. Proliferation before SIT (a), after one course of SIT (b) and after two courses of SIT (c) are compared.

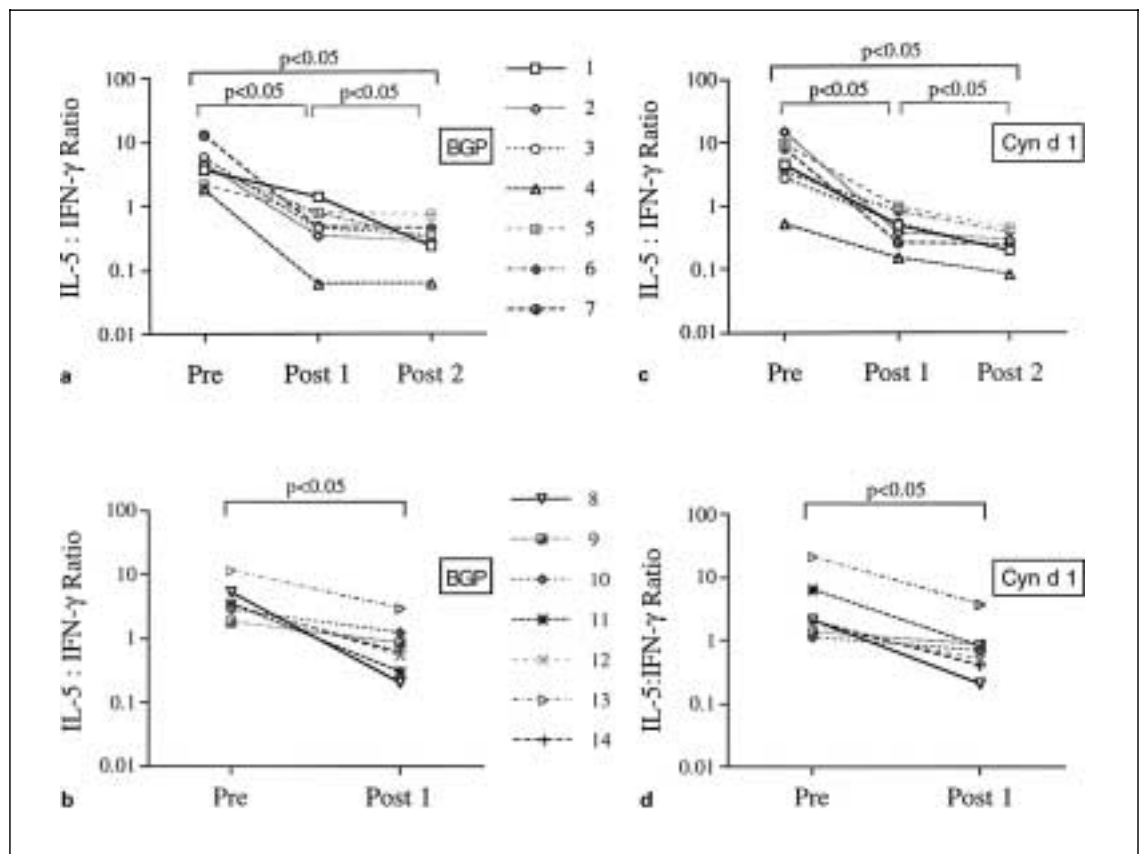


Fig. 6. Comparison of IL-5:IFN- γ ratios pre- and post-SIT (one or two courses) in BGP-allergic patients. Cytokine responses at allergen concentrations inducing optimal proliferative responses are used to calculate ratios.

that are primarily targeting the Pooideae subfamily (including rye grass and Timothy grass). Therefore, in the current study, BGP was added to the SIT preparation. All 14 patients reported a marked reduction in symptoms and medication use despite similar pollen counts for the seasons studied. This study permitted detailed analysis of the repertoire and function of human CD4⁺ T cells reactive with BGP and Cyn d 1 from atopic BGP-sensitised patients both before and after clinically effective SIT.

All BGP-sensitive patients studied showed strong T cell reactivity to a crude BGP extract. We demonstrated that the group 1 allergen of *C. dactylon*, Cyn d 1, is a major T cell allergen of BGP in addition to its role as a major B cell allergen. This study also investigated the specificity of human T cell responses at the epitope level, using overlapping peptides, demonstrating the presence of multiple epitopes located throughout the Cyn d 1 molecule. However, three highly immunogenic regions were identified, Cyn d 1 (109–128), (181–209) and (217–241),

that together elicited responses in 79% of the patients (11/14 patients), suggesting that these regions contain critical epitopes for an effective SIT preparation. Importantly, these three dominant regions show sequence identity in 11, 10 and 8 of the 12 reported Cyn d 1 isoforms, respectively [8, 24]. The effect of minor sequence differences between isoforms on T cell recognition of the dominant regions warrants investigation for vaccine development.

Seven patients were followed up for two courses of SIT treatment and 7 additional have completed one course of therapy to date. Underlying mechanisms of aeroallergen immunotherapy were also explored in this study. The clinically effective desensitization was accompanied by decreased T cell proliferation to BGP and Cyn d 1 in 79% of patients and decreased T cell proliferation to the Cyn d 1 peptides in all patients who were reactive with Cyn d 1. Importantly, there was negligible determinant spreading, as has been reported previously for grass pollen SIT using cloned T cells [25]. This will be of importance in future

refinements of SIT which target allergen-specific T cells. The response to an allergen would ideally be directed against only a limited number of antigenic determinants in order to provide a rational basis for the use of T cell-reactive sequences for desensitization.

Dysregulation of allergen-induced cytokine secretion with a marked reduction in the IL-5:IFN- γ ratio was observed in all patients. High levels of both cytokines were secreted pre-SIT, but post-SIT, the level of IL-5 secretion was markedly decreased. IFN- γ secretion was decreased in 10/14 patients post-SIT, but the degree of reduction was less compared to that for IL-5. Decreased production of IFN- γ , as well as IL-4/IL-5, has been reported following SIT for house dust mite allergy [26]. Studies reporting increased IFN- γ production following SIT were those where bee and wasp venom allergy were being treated [27–29], suggesting different mechanisms of SIT for an allergen naturally delivered by a sting compared with inhalation. The failure to promote a strong allergen-induced IFN- γ response by our BGP SIT protocol is a more attractive outcome in terms of avoiding possible IFN- γ -mediated pathology. There was no evidence of increased adverse reactions to SIT in our patients.

Overall, the findings of this study support anergy as a mechanism of successful SIT with decreased proliferation and attenuated cytokine production [30]. Although deletion of certain allergen-specific T cell subsets by high dose allergen treatment as would be experienced with SIT is difficult to exclude, anergy seems a more likely mechanism, since we could raise BGP-specific T cell lines from PBMC of SIT-treated patients albeit with altered reactivity. The persistence of an anergic T cell population with dysregulated cytokine production would provide a means for prolonging the benefits of SIT by providing a cytokine

milieu for deviating subsequent naïve allergen-specific T cell differentiation away from Th2 predominance.

The finding that Cyn d 1 was a major T cell allergen of BGP and the identification of three highly T cell reactive regions of Cyn d 1 in this study point to the feasibility of more refined BGP SIT preparations based on dominant T cell epitopes. Cyn d 1 (109–128), (181–209) and (217–241) were recognized by 57%, 43% and 43% of the patients pre-SIT, respectively. Further studies using truncated series of peptides are required to define the minimal T cell epitopes within these three regions. There was no clear association identified between MHC class II type and reactivity with one or more particular peptide sequences. This is consistent with the known high degree of promiscuity of peptide binding observed with MHC class II molecules [31, 32] and is not unexpected in view of the high level of clinical efficacy of current SIT achieved in an outbred human gene pool. Thus, the present study provides a strong basis for the development of more effective and safer BGP SIT preparations which target allergen-specific T cells. One approach is to use the dominant T cell epitope peptides themselves. Although there have been reported side-effects of peptide immunotherapy for cat allergy, these can be avoided by appropriate dosage and regimen development [33]. An alternative strategy is to use a modified form of Cyn d 1, in which T-cell-reactive sites are retained, but IgE-binding is ablated.

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