

Modification of T-cell receptor V β repertoire in response to allergen stimulation in peanut allergy

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Background: Peanut is one of the most common foods causing allergic reactions and is the most common cause of fatal and near-fatal food-related anaphylaxis. Little is known of the immunologic mechanisms that underlie peanut allergy.

Objectives: In this study we examined clonality of the T-cell response (TCR) to peanut in MHC class II identical, peanut allergy-discordant sibling pairs.

Methods: Four sibling pairs were investigated. The TCR repertoire was analyzed before and after in vitro stimulation of PBMCs with crude peanut or PHA, as control for general/non-specific reactivity. Eighteen TCR-V β families were examined by flow cytometry. Where significant differences in incidence of particular TCR-V β families were observed, PCR family-specific cDNA amplification and gene scanning were performed.

Results: After stimulation with peanut, no selective expansion of any TCR-V β subpopulation was observed with flow cytometry, in either the peanut-allergic or nonallergic siblings, with the exception of 1 peanut-allergic subject who demonstrated a significant increase of TCR-V β 11⁺ cells (0.3%-5.9% of the total CD3⁺ cells). However, gene scanning revealed predominant single-size PCR products for *TCR β V11* in all peanut-allergic subjects after peanut stimulation. *TCR β V11* polyclonality was observed in allergic and nonallergic subjects before peanut stimulation and in nonallergic subjects after peanut stimulation. In comparison, all subjects, before and after stimulation with peanut, showed polyclonality for *TCR β V2*.

Conclusions: Our results argue for clonal or oligoclonal TCRs to crude peanut and indicate that changes in the *TCR β V11* subpopulation are restricted to peanut-allergic subjects after stimulation with crude peanut allergen. (J Allergy Clin Immunol 2001;107:1089-94.)

Key words: Peanut allergy; T-cell clonality; TCR-V β families

Peanut is one of the most frequent foods causing allergic reactions in both children and adults and is the most common cause of fatal and near-fatal food-related anaphylaxis.^{1,2} The involvement of peanut in food-allergic reactions seems to have increased during the past decade. This may be due to an increase in infants' exposure to

Abbreviations used

TCR: T-cell receptor

SIs: Stimulation indices

SPT: Skin prick test

peanut in topical preparations as well as in foods.³ Another possibility is that peanut allergy is becoming more common as a reflection of the general increase in atopic diseases.⁴ Unlike many other food allergies, peanut allergy is rarely outgrown.⁵

Although considerable advances have been made in identifying and characterizing a number of peanut allergens,⁶⁻⁸ little is known of the genetic and immunologic mechanisms that underlie peanut allergy.

T cells recognize short peptides derived from intracellularly processed antigens in the context of cell surface MHC HLA molecules. Previous work from our laboratory has demonstrated a number of HLA class II associations with susceptibility to peanut allergy in case-control and family-based studies,⁹ demonstrating the importance of immunologic and immunogenetic factors in the development of peanut allergy.

The formation of the T-cell receptor (TCR) repertoire of mature circulating T cells is thought to be determined by both the genetic background of an individual and the response to environmental antigens.¹⁰⁻¹² Antigen-driven stimulation usually leads to clonal or oligoclonal expansions of T cells, with TCRs expressing common V, D, and J segments, whereas superantigenic stimulation leads to polyclonal expansion of a predominant TCR-V β family with different D and J segments and hence random antigen specificity.

In a recent study, selective expansion of T cells expressing a particular TCR- β chain variable gene segment (V β 2) was demonstrated after stimulation of peripheral blood lymphocytes of peanut-allergic individuals with crude peanut extract.¹³ However, in this study, expression of a limited number of TCR-V β families was examined in a small number of unrelated peanut-allergic and nonallergic individuals, without reference to HLA genotype. In addition, molecular genetic analysis of the clonal, oligoclonal, or polyclonal nature of these expanded TCR-V β 2 populations was not performed. Two other studies have demonstrated that peanut allergen-specific T-cell clones, isolated from the peripheral blood of

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TABLE I. HLA class II genotypes of sibling pairs

Pair	HLA class II genotype
1	DRB1*12, 07; DQB1*02, 0301; DPB1*0401, 0401
2	DRB1*04, 04; DQB1*0301, 0302; DPB1*0301, 0401
3	DRB1*01, 15; DQB1*0501, 0602; DPB1*0401, 0401
4	DRB1*16, 12; DQB1*06, 0301; DPB1*0301, 0402

peanut-allergic individuals, respond to the 2 characterized allergenic peanut fractions (Ara h 1 and Ara h 2) with a T_H2 cytokine profile, producing low levels of IFN- γ and high levels of IL-4.^{14,15} The presence of HLA class II restriction specificity of T-cell recognition of peanut allergens has been shown with clonal analysis.^{15,16}

In this study we sought to determine whether T-cell responses in peanut-allergic patients in vivo are specific to peanut (clonal or oligoclonal) or reflect general responses to allergens or lectins, acting as superantigens (polyclonal). For the first time, this investigation involved analysis of 18 TCR-V β families by flow cytometry in a series of HLA class II-matched sibling pairs discordant for peanut allergy. In addition, molecular gene-scanning clonality analysis was performed where significant differences in the incidence of particular TCR-V β families was observed before and after stimulation with peanut allergen, combined with molecular analysis of V β 2 T-cell populations.

METHODS

Subjects

Four HLA class II identical, peanut allergy-discordant sibling pairs were studied (peanut-allergic siblings: mean age 7.5 years, range 5-10 years; non-peanut-allergic siblings: mean age 5.0 years, range 3-7 years) and were identified in our previous study of HLA class II associations with peanut allergy.⁹ HLA class II genotypes of these pairs are given in Table I. Peanut allergy was confirmed by an unequivocal history of an immediate reaction to exposure to peanut, supported by appropriate positive/negative skin prick test (SPT) results to peanut. SPT results were considered positive if a wheal was elicited that was equal to or greater than the response to 1% histamine (positive control) in the absence of any response to 0.9% saline solution (negative control). Wheal diameters were greater than 3 mm, meeting the commonly accepted criteria for peanut allergy.¹⁷ Indeed, all subjects had wheal diameters of 5 to 6 mm. All non-peanut-allergic siblings had negative SPT results to peanut. Double-blind, placebo-controlled food challenges were not undertaken. This study received ethical approval from the Southampton and South West Hants Joint Research Ethics Committee (project No. 323/97).

TCR-V β expression analysis by flow cytometry

TCR-V β expression in total CD3⁺ cells in peripheral blood samples from the above HLA identical sibling pairs was examined before and after culture with crude peanut allergen and control mitogen (PHA). A panel of 18 FITC-labeled mAbs against TCR-V β family products 1, 2, 3, 5.1, 5.2/3, 6.7, 8, 11, 12, 12.1, 13.1, 13.1/3, 14, 16, 17, 20, 21.3, and 22 were obtained from Serotec (Oxford, UK) and Immunotech (Beckman Coulter, High Wycombe, UK).

Anti-CD3-phycoerythrin mAb was purchased from Becton Dickinson (Oxford, UK). Glutamine, penicillin, streptomycin, and RPMI 1640 were purchased from Gibco (Paisley, UK). Ten milli-

liters of venous blood was drawn from each patient; 100 μ L of whole blood was incubated in FACS tubes with saturating concentrations of anti-CD3-phycoerythrin and each one of the anti-TCRBV-FITC mAbs, in order to determine the percentage of CD3⁺ cells bearing each particular V β chain on their TCRs. These were considered baseline values.

PBMCs were isolated from the rest of the blood samples by density gradient centrifugation (Histopaque; Sigma Chemical Co, St Louis, Mo). The isolated PBMCs were resuspended in RPMI 1640 medium and adjusted to 1×10^6 cells per milliliter by counting of the cells with a Neubauer hemocytometer; 1×10^6 cells, supplemented with glutamine, penicillin, streptomycin, and amphotericin B, were cultured in Falcon tubes with PHA (1 μ g/mL) for 72 hours and with crude peanut allergen (100 μ g/mL) for 5 days separately at 37°C in a humidified atmosphere containing 5% CO₂.¹⁸ The 72-hour time culture of PBMCs with PHA diminishes the problem of the marked cell agglutination, which results from PHA activation and may be partly responsible for the decreased viability and number of cells recovered at 6 and 24 hours. Proliferation was determined by liquid scintillation counting of tritiated methylthymidine incorporation and stimulation indices (SIs) calculated as a ratio of the counts of cells stimulated with peanut or PHA over unstimulated cells.¹⁸

A 2-color immunofluorescence was performed with a FACScan flow cytometer (Becton Dickinson). A total of 15,000 events were collected, computed, and analyzed with appropriate software. An electronic gate was set on the lymphocytes on the forward and side scatter plot, and CD3⁺ cells falling within the gated area were then identified and selected into a second gate on the forward scatter (FSC)-FL2(CD3) plot. From 5000 to 7500 events were gated in the CD3⁺ area. V β expression was then analyzed by detection of FITC staining and expressed as a percentage of the total number of CD3⁺ cells. Baseline values, in both the nonallergic and the allergic siblings, were compared with the values estimated after stimulation with PHA and stimulation with peanut. TCR-V β cell expansions were defined as a value at least 2 times higher than the baseline value or any value over 15%. This value was selected to achieve comparability with other studies of T-cell expansions in allergic asthma.¹⁹

Molecular analysis of TCRBV gene families

From all the subjects included in the study, total RNA was extracted from PBMCs with a standard RNeasy B (Biogenesis, Poole, UK), chloroform-isopropanol-ethanol technique. Subsequently, cDNA synthesis with reverse transcriptase and an oligo dT primer (Not-I-d(T)₁₈) was made (First-Strand cDNA Synthesis Kit; Pharmacia, St Albans, UK) and kept at -70°C until use. For selected TCR-V β families, molecular analysis of the corresponding TCRBV gene family was performed by PCR BV family-specific sense primer and a 3' BC (constant region) antisense primer (5' fluorescence labeled), followed by gene-scanning analysis. Primer sequences were derived from the literature, and all protocols were previously established in our laboratory.²⁰ The PCR reaction mixture contained 1 to 2 μ L cDNA, 200 ng BV and BC primers, 200 μ mol/L of each dNTP, and 2 units of Taq polymerase (Boehringer Mannheim, Lewes, UK). Sterile water and 10 $\times$ PCR buffer were added to a total of 50 μ L. The samples were subjected to 33 cycles of PCR (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds). For analysis on the automated gene sequencer, 1 μ L of PCR product was mixed with 3 μ L formamide and 0.5 μ L of the internal size standard GeneScan-2500 Rox (Applied Biosystems, Warrington, UK). PCR products were denatured for 3 minutes at 90°C, size separated on a high-resolution polyacrylamide gel, and analyzed using the GeneScan 672 software (Applied Biosystems).

Fluorescent PCR products are represented as electropherograms, in which relative fluorescence intensities are plotted as a function of PCR product size. Products from clonal TCR-V β cell populations

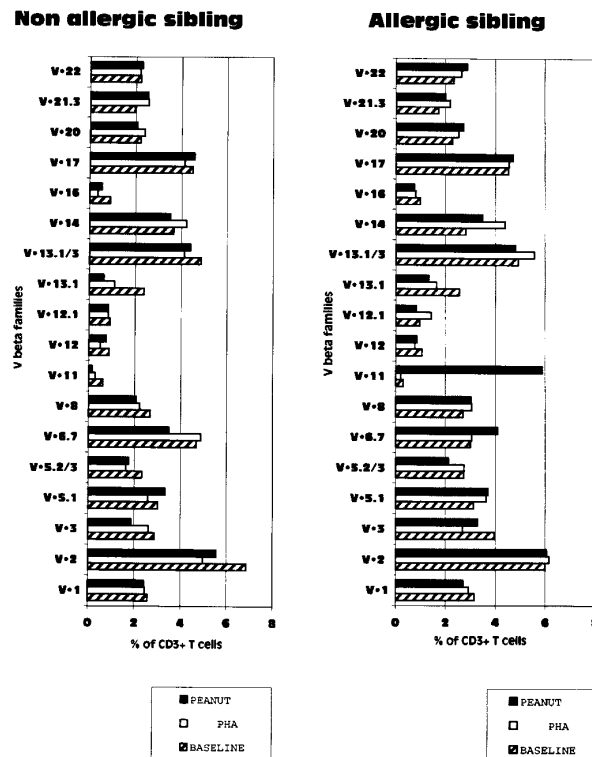


FIG 1. TCR-V β expression analysis of peripheral blood CD3⁺ T cells in peanut-allergic and non-peanut-allergic sibling pair 1, determined by flow cytometry.

were visualized as 1 or 2 sharp peaks of fluorescence corresponding to PCR-amplified clonal, rearranged TCR alleles, whereas cDNA from polyclonal TCR-V β cell populations was visualized as a fluorescence spectrum composed of polyclonal PCR fragments of different sizes in a normal Gaussian distribution with peaks spaced by 3 bp corresponding to selected in-frame *TCRBV* rearrangements. Oligoclonal profiles are defined as a modification of the Gaussian distribution, in which 3 or more distinct peaks appear above a polyclonal background.²⁰

This gene-scanning approach was followed for *TCRBV11* and *TCRBV2* for all subjects (allergic and nonallergic) after peanut allergen stimulation. Similar analyses were also performed for 2 sibling pairs before peanut allergen stimulation.

RESULTS

TCR-V β expression analysis by flow cytometry

PBMC cultures from both peanut-allergic and nonallergic siblings proliferated on stimulation with PHA, with SIs > 20. SIs for peanut were less (2-8), with a trend for greater proliferation in the peanut-allergic group. Proliferation was lower in the non-peanut-allergic group and not markedly higher than in previous studies by our group.¹⁸

In general, T cells from the peanut-allergic siblings did not demonstrate significant expansions of any particular TCR-V β subpopulation in the immunofluorescence analysis after stimulation with crude peanut. However, the TCR-V β 11 subset increased from 0.3% of the total CD3⁺ cells before stimulation to 5.9% after peanut stimulation in 1 peanut-allergic sibling (pair 1, Fig 1). In the non-peanut-

allergic siblings, none of the TCR-V β subsets showed any significant expansion compared with the baseline values after stimulation with peanut (eg, pair 1, Fig 1). After stimulation with PHA, no TCR-V β subpopulations were expanded in either peanut-allergic or nonallergic siblings.

Molecular analysis of *TCRBV* gene families

Gene-scanning analysis of the TCR-V β 11 subpopulation in the peanut-allergic sibling, who showed an increase from 0.3% before stimulation to 5.9% of the total CD3⁺ T cells after peanut stimulation in the immunofluorescence analysis, demonstrated the presence of a pronounced single-size PCR product after peanut stimulation, possibly indicative of a clonal TCR-V β 11 response to peanut allergen (Fig 2, A).

Subsequently, we performed molecular analysis of this particular TCR-V β 11 subpopulation in the other 3 peanut-allergic siblings, as well as in all 4 healthy (non-peanut-allergic) siblings after peanut stimulation. Although the immunofluorescence analysis did not demonstrate any significant increase of the TCR-V β 11 subpopulation in the 3 peanut-allergic siblings after peanut stimulation, gene-scanning analysis revealed the presence of pronounced single-size PCR products in all 3 peanut-allergic siblings, representing possible clonal or oligoclonal populations within the V β 11 family (for a representative subject, see Fig 2, B). Conversely, all non-allergic siblings showed multiple PCR product sizes, normally reflective of polyclonal populations.

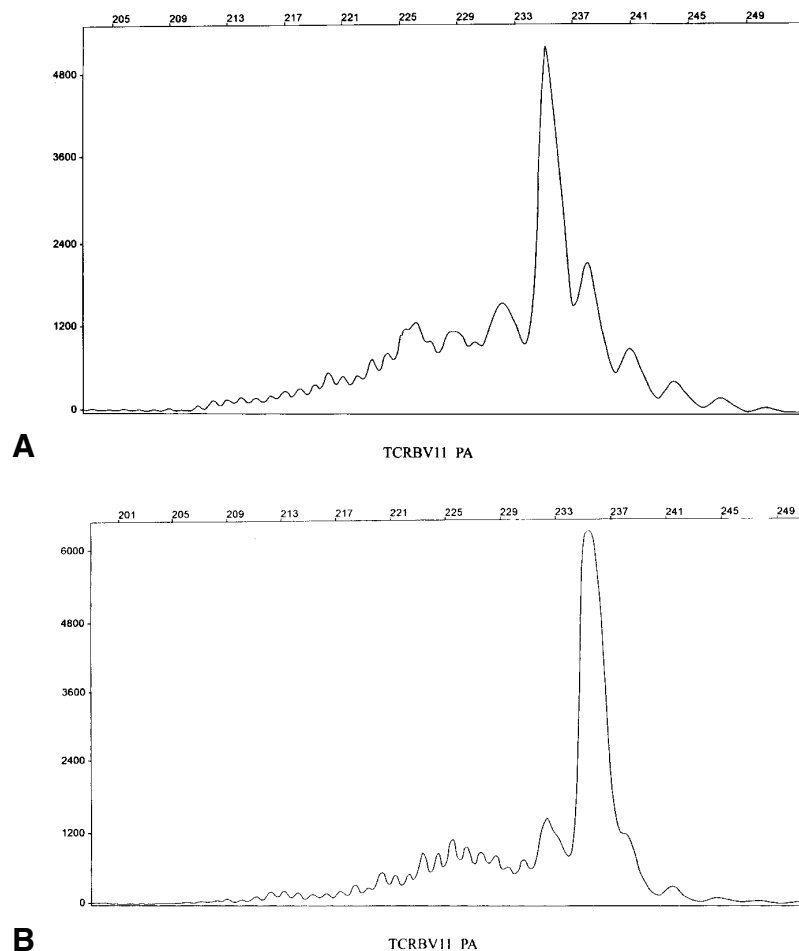


FIG 2. Molecular *TCRBV11* analysis of PCR products from cDNA from peripheral blood CD3⁺ T cells of peanut-allergic individuals after stimulation with crude peanut allergen, analyzed by gene scanning. **A**, Subject showing expanded TCR-Vβ11 CD3⁺ T-cell population after stimulation with crude peanut. **B**, Representative subject without expanded TCR-Vβ11 CD3⁺ T-cell population after stimulation with crude peanut allergen. Relative fluorescence intensities (*ordinate*) are plotted as a function of PCR product size (*abscissa*). PA, Peanut allergic.

Molecular analyses of TCR-Vβ2-expressing T cells in the peanut-allergic as well as in the nonallergic subjects after peanut stimulation were also performed because expanded TCR-Vβ2 populations had previously been reported after *in vitro* stimulation with peanut.¹³ In all cases, gene scanning showed PCR products of multiple sizes, reflective of polyclonal Vβ2 populations.

Finally, 2 sibling pairs were examined by gene-scanning analysis before peanut stimulation and polyclonality of the TCR-Vβ11 and the TCR-Vβ2 subpopulations were demonstrated in both the peanut-allergic and the nonallergic sibling, as evidenced by multiple PCR products of different sizes, as revealed by gene scanning.

DISCUSSION

We investigated the specificity of T-cell responses to peanut in peripheral blood CD3⁺ T cells before and after stimulation with crude peanut allergen. The T-cell proliferative

response to a general mitogen (PHA) was also examined as control for general/nonspecific reactivity. Our study was restricted to HLA identical, peanut allergy-discordant sibling pairs, to avoid the confounding effects of possible HLA influences on the expressed peripheral blood TCR repertoire, which remain equivocal.^{11,21,22}

In this study a significant expansion of T cells expressing TCR-Vβ11 was seen only in 1 peanut-allergic subject after peanut stimulation. No selective expansion of any other TCR-Vβ subpopulation was observed with immunofluorescence analysis in either the peanut-allergic or the nonallergic siblings. Because it is known that oligoclonal or clonal expansions in response to an antigen can occur in the absence of a family-specific TCR-Vβ expansion, our findings in the immunofluorescence analysis do not exclude the presence of such oligoclonal or clonal responses after stimulation with crude peanut. Indeed, molecular analysis and gene scanning revealed

predominant single-size PCR products for *TCR β V11* in all 4 peanut-allergic subjects after peanut stimulation, including the 3 subjects who did not show an increase in TCR-V β 11 expression with flow cytometry. In a mixed T-cell population, the frequency of antigen-specific T cells is generally low, and this may account for the lack of detectable expansion of TCR-V β 11 subpopulation in 3 of 4 peanut-allergic subjects after peanut stimulation in the immunofluorescence analysis. These results do not provide definitive evidence for clonal *TCR β V11* populations in these subjects (confirmation by sequencing is required). Nevertheless, our finding of pronounced shifts in the *TCR β V11* repertoire in all peanut-allergic subjects after peanut stimulation has greater importance in view of the predominant TCR-V β 11 polyclonality observed in these subjects before peanut stimulation and in all nonallergic subjects after peanut stimulation. This indicates that changes in the *TCR β V11* repertoire detectable by gene scanning are restricted to the peanut-allergic subjects after stimulation with crude peanut allergen. No selective expansion of V β 2 was seen in any subject, unlike that seen in the study by Dorion and Leung,¹³ although the technical details of our T-cell stimulation protocols differ somewhat. Moreover, the peanut-allergic subjects gave *TCR β V2* gene scans consistent with polyclonal lymphocyte populations both before and after peanut stimulation, at the level of sensitivity of this technique. However, it should be noted that V β 2 was the most frequent T-cell subset at baseline in most subjects examined. Conversely, the TCR-V β 11 subset was smaller than the V β 2 subset. However, taken together, our results suggest that the response of the TCR-V β 11-expressing subpopulation to peanut allergen in peanut-allergic subjects is most likely antigen specific. Although we performed gene-scanning analysis only for 2 *TCR β V* families, it is important to note again that apparent clonality was revealed in the TCR-V β 11-expressing subpopulation and was observed only in the peanut-allergic subjects and only after peanut stimulation. This is an important finding because PBMCs from both peanut-allergic and nonallergic siblings proliferated in response to crude peanut (some of this response may be due to peanut lectins). Lectins are not known to induce V β -specific T-cell expansion, and in accordance with this, after PHA stimulation, no expanded TCR-V β families were detected, in either peanut-allergic or non-peanut-allergic subjects, despite strong T-cell proliferation in response to PHA.

Recently, we showed that particular HLA class II genotypes show highly significant associations with peanut allergy, clearly demonstrable even in light of SPT positivity to crude peanut allergen.⁹ This finding may also argue for clonal or oligoclonal T-cell responses to immunodominant epitopes expressed on crude peanut allergens in vivo. In this study 2 of the sibling pairs, including that (pair 1) in which the allergic sibling showed a selective expansion of TCR-V β 11-expressing T cells, carried a DRB1 allele encoding the Tyr16 residue previously shown to be associated with peanut allergy.⁹ A third pair carried the DPB1*0301 allele, also signifi-

cantly associated with peanut allergy in our previous study.⁹ The final sibling pair studied was not of a peanut allergy-associated genotype. On the basis of this small sample, there is no distinguishing feature of the HLA class II genotype of the peanut-allergic sibling who showed a TCR-V β 11 expansion after in vitro stimulation with crude peanut.

Numerous studies have also reported variable associations between particular HLA class II alleles and IgE responses to specific allergens, the most consistent of which is between HLA DRB1*15 and the low-molecular-weight ragweed allergen Amb a 5.²³ To date, much of the research in food allergy has concentrated on IgE responses to specific allergens. However, the results presented in this preliminary study argue for clonal or oligoclonal T-cell responses to immunodominant epitopes expressed on crude peanut allergens in vivo. This is remarkable in a crude allergen such as peanut, which expresses potentially numerous epitopes with multiple HLA class II DR, DQ, and DP restriction elements. Further studies are therefore indicated in an independent patient series and unrelated control group, using purified Ara h1 and Ara h2 allergens and an unrelated allergen or allergens, in addition to crude peanut, to confirm these findings. Such an investigation should also include studies of T-cell function in terms of cytokine production of clonally expanded TCR-V β subsets in peanut-allergic subjects.

In conclusion, this study provides evidence for clonal or oligoclonal T-cell responses to crude peanut and indicate that such changes in the *TCR β V11* subpopulation are restricted to peanut-allergic subjects after stimulation with crude peanut allergen. On the basis of these findings, further studies in allergic and nonallergic subjects are indicated to elucidate the immunologic mechanisms of this most severe form of food allergy.

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