

Peanut allergic subjects' peripheral blood mononuclear cell proliferative responses to crude peanut protein

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Summary

Background Peanut allergy is characterized by a high frequency of severe and occasionally fatal reactions.

Objective To determine if there are features of the *in vitro* cellular response that may account for the observed severity of peanut allergy.

Methods Skin-prick testing (SPT), RAST assay of serum peanut-specific and total IgE and mixed peripheral blood mononuclear cells (PBMC) proliferative responses to crude peanut protein were measured in 44 peanut allergics with varying severity of clinical reactions. PBMC responses of 13 non-peanut allergic controls (six atopic) were also studied.

Results Subjects' PBMCs proliferated more than controls', even without stimulation. Subjects' PBMC proliferative responses did not correlate with clinical severity, SPT wheal size or peanut-specific IgE levels. Controls' PBMCs did not respond to peanut. There was no correlation between PBMC response and time since last reaction to peanut. Subjects' PBMCs responded more than controls, to mitogen as well as allergen. Proliferation increased with increasing concentration of peanut protein ($P < 0.0001$).

Conclusion PBMCs of peanut allergics demonstrate a dose-dependent response to peanut which does not correlate with clinical severity, SPT reaction or levels of peanut specific IgE. The response is antigen-specific. Peanut protein is not mitogenic and is not acting as a superantigen. While there are non-specific differences in the PBMC responses of peanut allergic individuals compared with atopic and non-atopic controls, these differences do not explain the unique severity of peanut allergy.

Keywords: peanut allergy, T cell responses, anaphylaxis

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Introduction

Food allergy affects up to 8% of children [1] and reactions to peanut are the most common cause of fatal and near-fatal food-related anaphylaxis in both adults and children [2,3]. The prevalence of peanut allergy is increasing — recent figures suggest 0.6% of British 4-year-olds are peanut allergic and an additional similar number are sensitized but have had no clinical reactions to peanut [4]. The number of referrals for evaluation of peanut allergy has doubled in the last 10 years [5]. Unlike allergies to cow's milk or egg, peanut allergy frequently persists into adulthood [6]. There

are few data on food allergy relating clinical severity to IgE levels [7] or peripheral blood mononuclear cell (PBMC) responses [8].

It is possible that the generally severe phenotype of peanut allergy is influenced by a combination of factors such as a mitogenic effect of peanut proteins, an exaggerated allergenicity of peanut proteins, hyperreactivity of sensitized T cells to peanut protein or a higher frequency of circulating peanut-sensitized T cells. Dorion and Leung have proposed that restricted use of the $V\beta_2$ T-cell receptor (TCR) may account, partly, for the severity of the immune response to peanut [9]. Studies have confirmed the presence of a TH_2 phenotype in peanut allergic subjects' responses to one of the major antigens of peanut, Ara h 2 [10], and the presence of HLA class II restriction specificity of T-cell

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recognition of peanut allergens, using clonal analysis [11,12]. We have recently shown an overrepresentation of certain HLA class II genotypes in peanut allergic subjects and their families, compared to unrelated controls [13].

To identify whether cellular factors contribute to the severity of peanut allergy we have investigated the PBMC proliferative responses to crude peanut protein in 44 peanut allergic patients and studied the correlation of PBMC proliferation with clinical severity, skin-prick test (SPT) weal size and both total and peanut-specific serum IgE levels. We have compared PBMC proliferative responses with the mitogen phytohaemagglutinin (PHA) and anti-CD3, the cow milk allergen, beta-lactoglobulin (BLG) and three concentrations of crude peanut extract (CPE).

Materials and methods

Subjects

Forty-four peanut allergic patients were studied (30 children, 21 male mean age 4.25 years, range 1–14 years and 14 adults, four male, mean age 28.5 years, range 15–37 years). Peanut allergy was confirmed by personal interview revealing an unequivocal history of reaction to an exposure to peanut, supported by positive skin-prick tests (SPT) with peanut (1:10 w/v peanut mix [Runner, Virginia, Spanish] Miles Inc., Indiana, USA). SPT was considered positive if it elicited a weal equal to or greater than the response to 1% histamine (positive control) in the absence of any response to saline 0.9% (negative control). Double-blind, placebo-controlled food challenges (DBPCFC) were not undertaken, but we have shown in open challenges of subjects reporting peanut allergy that the false positive rate of peanut SPT is only 13% [14]. The clinical details and SPTs with other allergens of some of the children involved in this study have been published elsewhere [15].

Twenty-six subjects (59%) had asthma, 30 (68%) eczema and 17 (38%) seasonal and/or perennial rhinitis. Allergy to tree nuts was reported by 17 subjects (38%); to milk by two subjects (4.5%); to egg two subjects (4.5%), to legumes (other than peanut) two subjects (4.5%); fish two (4.5%), fruit one (2.25%) and wheat one (2.25%).

Six subjects (13.6%) had mild peanut allergy (rash or urticaria), 21 (47.7%) had moderate peanut allergy (ranging from facial oedema to laryngeal oedema) and 17 (38.6%) had severe symptoms such as wheeze, cyanosis or fainting/collapse.

Twenty-five subjects (56.8%) had suffered a reaction in the previous year, 11 of which had been in the preceding 6 months. Two subjects (4.5%) had not had a reaction for more than 5 years. Seventeen subjects (38%) had ever attended hospital for emergency treatment of a reaction to peanut and only five (11.4%) had ever received or self-administered injected adrenaline.

Controls

Ten adult controls were studied (five male). Five controls (two male) were atopic but none was food allergic and none had a positive SPT or RAST to peanut. Three subjects who reported peanut allergy but had negative SPTs and negative open challenges [15] were also studied. One of these subjects had a positive SPT to grass pollen and is included in the 'atopic control' group. The other two peanut challenge negative subjects had no other manifestations of atopy and are included in the 'non-atopic control' group. Blood was only taken from children at the same time as clinically indicated tests.

Radioallergosorbent (RAST) measurement of serum peanut specific IgE was performed using a commercially available liquid allergen based micro titre plate ELISA system (Euro/DPC Ltd, Llanberis, UK).

Isolation of mixed PBMCs

Venous blood (5–10 mL) was collected over heparin. Peripheral blood mononuclear cells were separated by density gradient centrifugation on Lymphoprep (Nycomed Pharma AS, Norway) [16] for 25 min at 4°C at 2000 rpm. Cells were resuspended in RPMI 1640 medium (Gibco Life Technologies, UK) supplemented with 2 mmol of L-Glutamine, 100 IU/mL of penicillin and 100 mg/mL streptomycin.

Proliferation assay

Mixed PBMCs (10^5 /well and 125 μ L of autologous plasma/well) were cultured in 5% CO₂ at 37°C in round bottomed 96-well micro-titre plates (Costar, Cambridge, MA, USA). Cultures were undertaken in triplicate with negative control (cells incubated in same volume of culture medium without mitogen or antigen — 'unstimulated' cells). 'Stimulated' cells were incubated with either mitogen or antigen. Mitogenic proliferation was studied in response to phytohaemagglutinin (PHA, 0.1 mg/mL, Sigma Chemicals, Poole, UK) and, in a subgroup of patients, anti-CD3 (25 μ m/mL, Ortho Diagnostics, USA). To investigate whether the response to PHA was due to it being a plant lectin, and therefore similar to some peanut proteins, the response to the mitogen anti-CD3 was studied in six peanut allergic subjects. These results were compared to those of six atopic and seven non-atopic adult controls generated by another study in the department under identical, but not contemporaneous, experimental conditions. These control results are reported here with the kind permission of Dr Mandy Jones.

The antigenic response to the unrelated food allergen betalactoglobulin (BLG, 1 mg/mL, Sigma), a major allergenic component of cow's milk, was also studied. The cells

were cultured with concentrations of crude peanut protein (CPE, Sigma) of 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL. These concentrations span some of those used by de Jong who showed a dose-dependent proliferative response over concentrations from 1 to 250 µg/mL [12].

Cultures were pulsed with tritiated methylthymidine (1 µmCi/well)[³H]TdR; Amersham International, Amersham, (UK) after 3 days in the case of mitogen and after 6 days in the case of antigen and were harvested 16 h later. These times for pulsing and harvesting have been shown in this laboratory [18] and others [19] to be optimal for studies of PHA and antigens. Proliferation was determined by liquid scintillation counting of [³H]TdR incorporation. Results are expressed either as counts per minute (cpm) or as a ratio of the counts of cells stimulated with mitogen or antigen over unstimulated (negative control) cells. A ratio (SI) of greater than three to one was considered significant.

Statistical methods

Laboratory data were analysed using Arcus Professional Statistical Analysis Software, version 2.03 A (copyright IE Buchan, 1992) and SPSS for Windows, version 6.1 (SPSS, Chicago, USA). The Mann–Whitney *U*-test was used to compare means of non-parametric data from independent groups. Spearman's rank correlation (expressed as the coefficient rho, ρ) and the Wilcoxon signed rank test were used to evaluate correlation of nonparametric data on individual groups. Kruskal–Wallis test was used for one way analysis of variance of non parametric data. Two-tailed *P*-values and 95% confidence intervals were used for all data analysis.

Ethical approval

The study was approved by the Southampton Joint Ethics subcommittee and written personal or parental informed consent was obtained from each subject and control.

Results

Median peanut SPT weal size was 6 mm (range 3–15 mm). Median peanut RAST score was 3[0 1,2,3,4,5,6], and median total IgE was 116 IU/mL (5–1200 IU/mL). This low median total IgE may reflect the predominance of young children in the group. Seven subjects had a RAST of 0. These subjects had a range of positive SPT reactions to peanut of 3–10 mm. Five of these were under 2 years of age. Two had mild reactions, three moderate and two severe reactions, by history.

Proliferative response of unstimulated cells

There was a significant difference between the absolute counts of unstimulated cells of subjects (median 767 cpm, range 200–124 420) and controls (median 286, range 203–1800, Mann–Whitney *U*-test, *P* = 0.03), see Table 1. In peanut allergic subjects there was no significant association between clinical severity and counts of unstimulated cells (Spearman's coefficient ρ = –0.009, *P* = 0.96).

PBMC responses to mitogen and allergen

Responses to the mitogens PHA and anti CD3 and the allergens BLG and CPE were significantly greater than

Table 1. Proliferative responses of peanut allergics and controls to mitogen and antigen

	Peanut allergic median cpm (range)	Peanut allergic median SI	Control median cpm	Control median SI	Mann–Whitney SIs <i>P</i> -value
Unstim	767 (200–124 420)		286 (203–1802)		0.03
PHA	4304 (20–292 338)	7.54 (3.1–79)	934 (430–20 764)	5.0 (2.5–10.6)	0.03
BLG	1162 (166–118 771)	1.5 (0.2–6.7)	488 (245–3429)	1.7 (0.57–4.2)	0.8
CPE 0.01	2310 (274–298 700)	4.23 (0.7–26.4)	309 (170–2239)	1.2 (0.72–1.52)	<0.0001
CPE 0.1	4871 (590–360 964)	5.86 (1.49–35.4)	526 (288–967)	1.4 (0.43–2.86)	<0.0001
CPE 1.0	6150 (892–552 728)	8.5 (2.53–46)	598 (337–1124)	1.66 (0.62–2.84)	<0.0001

the counts of unstimulated T cells for both peanut allergic subjects and controls (Wilcoxon signed rank, matched pairs, $P < 0.0001$ in every case). The proliferative responses to either mitogen or allergen of the two control groups did not differ significantly and the two control groups have been combined for further analysis, except where specified.

PBMC response to mitogen (PHA and anti-CD3)

There was a response to PHA in all groups (Fig. 1) but peanut allergics' PBMCs were significantly more responsive to PHA than were controls (subjects' median SI 7.54 [3–79.16] vs controls 5.0 [2.5–10], Mann–Whitney U -test, $P = 0.03$), see Table 1. There was no correlation between PBMC response to PHA and clinical severity (Kruskal–Wallis 1-way ANOVA $\chi^2 = 2.0$, $P = 0.37$).

The mean proliferative response (SI) of peanut allergics to anti CD3 was 16.07 (SE 4.03). Non-atopic controls' mean response was 19.32 (SE 6.09) and atopic controls' mean response was 44.33 (SE 25.86). There was no difference between subjects' and the combined controls' responses to anti-CD3 (Mann–Whitney, $P = 0.6$). There was no difference between subjects' responses to PHA and anti CD3 (Wilcoxon Matched pair, $Z = -0.15$, $P = 0.91$). The response to mitogen was similar whether due to the plant derived PHA or to anti-CD3.

PBMC response to beta-lactaglobulin

There was no difference between subjects' and controls' responses to BLG (SIs 1.5 (0.2–6.7) vs 1.7[0.57 4.26], Mann–Whitney U -test, $P = 0.8$).

PBMC response to peanut

In peanut allergic subjects there was a dose–response relationship with statistically significant increases of T-cell proliferation between the peanut concentrations of 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL (Wilcoxon matched-pairs signed-ranks, $P < 0.0001$, and $P < 0.0001$, respectively, see Table 1 and Fig. 2). PBMCs of atopic and non-atopic controls did not respond significantly to peanut protein at the concentrations tested (Fig. 3). Peanut allergic PBMC responses were significantly greater than those of combined controls at each peanut protein concentration — median SIs 4.23 vs 1.2 at 0.01 mg/mL (Mann–Whitney U -test $P < 0.001$); at 0.1 mg/mL 5.86 vs 1.4 ($P < 0.001$) and at 1 mg/mL 8.5 vs 1.66 ($P = 0.0001$), respectively. Amongst peanut allergics there was no association between the slope of the PBMC dose–response curve to CPE and either clinical severity (Kruskal–Wallis 1-way ANOVA $\chi^2 = 1.15$, $P = 0.56$) or between PBMC response and time since last

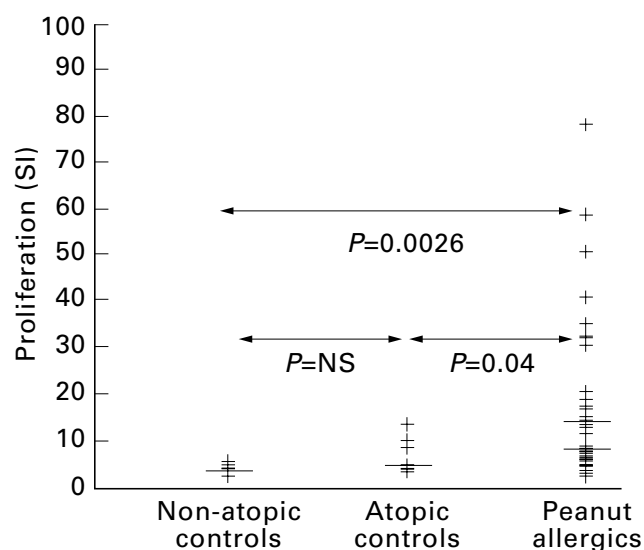


Fig. 1. Proliferative responses of controls' and peanut allergics' PBMCs to PHA.

exposure to peanut (Kruskal–Wallis 1-way ANOVA $\chi^2 = 3.45$, $P = 0.49$, Fig. 4).

There was no difference in PBMC responses to 1 mg/mL CPE when peanut allergic subjects were classified according to severity of reported clinical reaction to peanut (Spearman's rank correlation coefficient, $\rho = -0.1178$, CI -0.33 – 0.27 , $P = 0.45$), to peanut SPT weal size ($\rho = -0.22$, CI -0.49 – 0.08 , $P = 0.15$), RAST ($\rho = 0.25$, CI -0.07 – 0.51 , $P = 0.12$), or total serum IgE concentration ($\rho = 0.15$, CI -0.18 – 0.45 , $P = 0.38$).

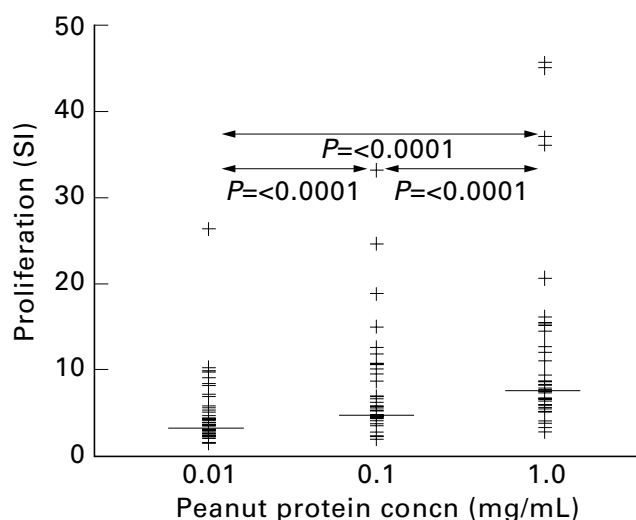


Fig. 2. Proliferative responses of peanut allergics' PBMCs to crude peanut protein (CPE).

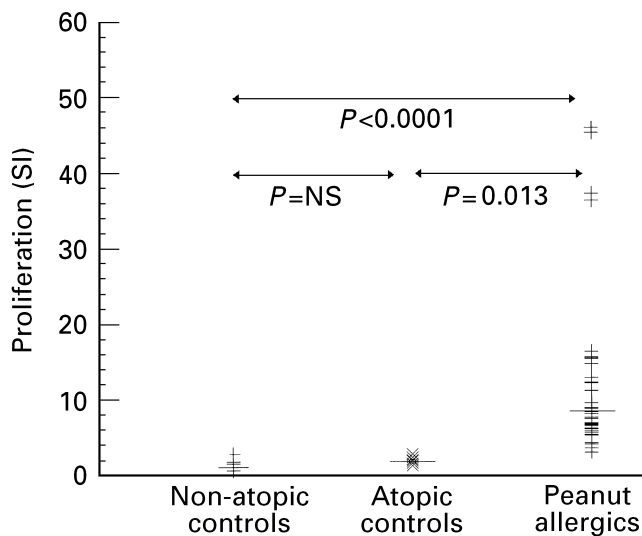


Fig. 3. Proliferative responses of controls' and peanut allergics' PBMCs to crude peanut protein (1.0 mg/mL).

Discussion

Peanut allergy is characterized by a higher frequency of severe and fatal reactions than is found in other food allergies [2,3]. Our study of 44 unselected peanut allergics shows that neither the simple *in vivo* test (skin-prick testing) nor RAST assay of peanut-specific IgE correlated with the severity of reported reaction. Examination of an earlier step in the allergic pathway — T-cell proliferative responses — did not reveal any factor predictive of clinical reactivity. The proliferative response of peanut allergic subjects' PBMCs to crude peanut protein was unrelated to the time interval from the last exposure to testing and did not account for the observed frequency of severe reactions to peanut.

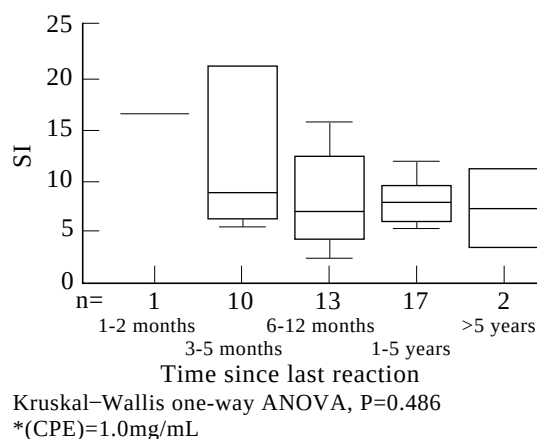


Fig. 4. Proliferation in response to peanut vs time since last reaction.

Other workers have shown that the severity of reactions to peanut is not due to a mitogenic effect of peanut protein [10,11]. This is confirmed by our study, where controls (atopic and non-atopic) did not respond significantly to peanut protein at the concentrations tested. Peanut protein did not stimulate proliferation of unsensitized T cells in controls and clinical severity correlated neither with counts of unstimulated PBMCs (higher in peanut allergic subjects than controls) nor with response to CPE. HLA class II restriction of T-cell recognition of peanut proteins [11] suggests that a superantigen effect is not a major factor in allergic reactions to peanut.

Peanut allergics' PBMCs responded to PHA significantly more than the PBMCs of atopic controls, most of whom had allergies to inhalant allergens to which they were constantly or frequently exposed. A plant derived lectin such as the minor peanut allergen peanut agglutinin PNA can act as a mitogen to bovine PBMCs [20] but this appears not to be a significant factor in the response to crude peanut extract for two reasons. de Jong has shown firstly that PNA alone stimulates peanut allergic PBMCs only weakly and secondly that PNA does not stimulate control PBMCs significantly [12]. Our study confirmed that control PBMCs did not react to CPE. Peanut allergics' PBMCs responded specifically to peanut antigen. The response was antigen specific, with no difference between subjects (only two subjects reported milk allergy) and controls in response to the milk protein BLG. Responses to peanut by peanut allergics' PBMCs were greater than the responses of egg allergics, PBMCs to ovalbumin [21].

The higher rate of proliferation by PBMCs of peanut allergics compared to controls in the unstimulated state and after stimulation with PHA and antiCD3 (disregarding their allergen-specific response to CPE) suggests there may be some intrinsic excessive reactivity of peanut allergics' T cells, even in the resting state, possibly due to the unavoidable, frequent exposures to peanut [7,22]. Such frequent high-dose, but intermittent exposure may repeatedly up regulate peanut sensitised T cells in contrast to down regulation by low dose constant exposure of asthmatics to inhalant allergens. However, even in subjects who successfully avoided peanut for relatively long periods, significant proliferative responses were seen.

There are no data on the relative expression of markers of memory T cells ($CD45RO^+$) and of activated T cells ($CD25$) in subjects allergic to different foods. Due to the frequency of exposure to peanut of peanut allergics [7,22], — and by inference the general population — a most interesting study would a longitudinal study of the developing T-cell populations and their proliferative responses in individuals (children) known to be sensitized but without a history of an allergic reaction to peanut [4].

This unselected group of 44 peanut allergics contained only six subjects with a history of mild reactions to peanut. This uneven distribution may account for the lack of correlation of clinical severity with PBMC responses. This study has shown that peanut allergic PBMCs react significantly to mitogenic as well as antigen specific stimulation but that these reactions do not correlate with clinical reactivity to peanut. Other factors in the allergic response may be more important. Further studies of HLA class II restriction and TCR expression in large groups of peanut allergics and further studies of proliferative responses to isolated and purified peanut proteins are required to elucidate the cellular mechanisms of this most severe food allergy.

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