

T cell epitopes of the major fraction of rye grass *Lolium perenne* (*Lol p I*) defined using overlapping peptides *in vitro* and *in vivo*. I. Isoallergen clone1A

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SUMMARY

One hundred and fifteen overlapping synthetic peptides spanning the entire sequence of the isoallergen clone1A of *Lol p I* from rye grass *Lolium perenne* were synthesized by the multi-pin technique. The peptides were overlapping 12mers, offset by two residues and overlapping by 10 residues. Sets of six adjacent overlapping peptides (except pool-1,15,20) were pooled and were used *in vitro* and *in vivo* to map the T cell epitopes on *Lol p I*. Six atopics who were skin test and RAST positive to rye grass showed T cell responses to *L. perenne* extract (LPE) and its major fraction (*Lol p I*). Five out of six showed T cell responses *in vitro* to peptide pool-17, while five non-atopics did not respond to any of the peptide pools. By testing the individual peptides of pool-17, we have located the T cell epitope on *Lol p I*. Interestingly, when we tested pool-17 and its single peptides *in vivo* by intradermal skin testing we found in one patient a typical DTH after 24-48 h to pool-17 and its peptides (peptides 3 and 4) which exactly matched the *in vitro* responses. By defining the T cell epitopes in this way a greater understanding of the allergic response to pollen will be obtained, and a more effective and less dangerous vaccine may be possible for treating patients with hay fever.

Keywords T cell epitope rye grass *Lolium perenne* Delayed type hypersensitivity
synthetic peptides hay fever

INTRODUCTION

Allergic diseases affect an increasing proportion of the world's population, with symptoms ranging from mild conjunctival irritation to life-threatening conditions and even death [1]. Common environmental allergens such as grass pollen, house dust mite or animal dander are the major causes of symptoms. Inhalation of pollen in the atmosphere evokes IgE-mediated responses typical of hay fever and pollen asthma, which can affect up to 20% of the population [2,3]. In 915, Razi gave an explanation of head colds as due to allergy or what is now known as hay fever [4]. Hay fever has become an increasing problem in the Western hemisphere [5] and in other countries [6].

Immunotherapy as treatment has been used since 1911 [7] to reduce the susceptibility of patients to environmental allergens. However, all desensitizing agents have the potential to induce allergic type reactions, the most serious of which are bronchospasm, anaphylaxis and even death. In the period from 1957 to

1986, 26 patients in the UK died from anaphylaxis induced by the desensitizing vaccines, putting into doubt the safety of such vaccines. This led to a report by the UK Committee on the Safety of Medicines, which placed restrictions on the use of allergen vaccines.

One of the major causes of hay fever is the pollen of rye grass *Lolium perenne*, which is one of the most common allergens [3]. Several proteins from this grass pollen have been isolated, and the complete amino acid sequences for three of these proteins, *Lol p I* [8,9], *Lol p II* [10] and *Lol p III* [11] have been published. Most grass-sensitive patients (95%) possess specific IgE antibodies to the major fraction of *L. perenne* (*Lol p I*) [12], which is a heterogeneous protein with mol. wt between 27 and 35 kD. The heterogeneous forms of *Lol p I* have been termed isoallergens [13,14]. The complete amino acid sequence of the isoallergen clone1A of *Lol p I* was deduced from cDNA sequence analysis [8].

T helper cells have a crucial role in the control of IgE synthesis, and in view of our earlier study with crude pollen extracts showing delayed skin test reactivity in atopic subjects with hay fever [15] we wished to define the specific T cell epitopes of *L. perenne*. We have not used T cell clones from atopics in

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Table 1. Characteristics of the atopic (AS) and non-atopic (NAS) subjects participating in the epitope mapping study

Subjects	Skin prick test		Total IgE, U/ml	RAST class specific IgE level	History
	LPE	<i>Lol p I</i>			
AS1	+	+	> 500	2	Atopic
AS2	+	+	170	3	Atopic
AS3	+	+	370	4	Atopic
AS6	+	+	> 500	4	Atopic
AS8	+	+	180	4	Atopic
AS26	+	+	> 500	4	Atopic
NAS1	—	—	< 150	0	Non-atopic
NAS2	—	—	< 150	0	Non-atopic
NAS3	—	—	< 150	0	Non-atopic
NAS4	—	—	< 150	0	Non-atopic
NAS14	—	—	< 150	0	Non-atopic

LPE, *Lolium perenne* extract.

order to map the epitopes, as we felt this would not accurately reflect the number and specificity of the T cells in the circulating lymphocyte pool. For this reason, we cultured peripheral blood mononuclear cells (PBMC) with peptide. By using all possible 12mer overlapping synthetic peptides homologous with the sequence of the iso-allergen clone1A [8] of *Lol p I*, we have defined linear peptides of this major grass pollen allergen which stimulate lymphocytes from hay fever patients *in vitro*. Patients were also skin tested, looking for DTH using the same peptide pools that were recognized *in vitro*. With one of six patients tested, the peptides that were active *in vitro* did produce a DTH reaction on skin testing *in vivo*.

Our data showing the T cell epitope of the major rye grass allergen may allow the development of safer and more efficacious vaccines.

SUBJECTS AND METHODS

Ethical approval

Full ethical approval was obtained from the Middlesex Hospital, London, UK, before the start of this study.

Subjects

Six atopic volunteers who participated in this study were recruited from the Allergy Clinic, Middlesex Hospital. They were skin test and RAST positive to *Lol p I* and whole pollen extract. The characteristics of the six atopic rye grass-sensitive patients and five non-atopic subjects are shown in Table 1. All the atopics had relevant seasonal symptoms. These patients had not received immunotherapy. Over and above being atopic, the patients had to live or work within a reasonable distance of the clinic because of the necessity for extensive skin testing and also repeat venesection. Six atopic subjects are dealt with in this study (five male, one female) aged 28–55 years, and five non-atopic (three male, two female) aged 28–45 years.

Preparation of *Lol p I*

Rye grass (*L. perenne*) pollen extract was supplied by Dome Laboratories (Stoke Court, UK). Following 33% ammonium sulphate precipitation, the supernatant was dialysed against

PBS. The dialysed sample was injected on to fast performance liquid chromatography (FPLC; Pharmacia LKB, Uppsala, Sweden) for protein separation on a gel filtration column Superose 12, and 0.3-ml fractions were collected. By measuring the optical density (OD) at 280 nm, two peaks (peaks 1 and 2) were identified.

Gel electrophoresis

SDS-PAGE was performed on a vertical slab-gel apparatus (Bio-Rad, Richmond, CA). Fifteen per cent gradient acrylamide gels were employed in this study and run at 180 V for 12 h. The apparent molecular mass of the allergens was estimated by using prestained protein standards.

Synthetic peptides

A set of 115 overlapping, 12mer peptides spanning the entire length of the iso-allergen clone1A [8] of the *Lol p I* was synthesized using the multi-pin technique, which enables the rapid mapping of T cell determinants [16]. The peptides were cleaved from the pins using 0.1 M phosphate buffer (pH 7) for 4 h at room temperature. The purity of selected peptides, including all peptides in pool 17, was established by reverse phase high performance liquid chromatography (HPLC) analysis and the amino acid composition was checked by amino acid analysis (Cambridge Research Biochemicals Ltd. and Chiron Mimotopes Pty. Ltd.). The iso-allergen clone1A of the *Lol p I* consists of 240 amino acids [8], and sets of six continuous peptides were pooled, with the exception of pools 1 and 15 (five peptides) and pool 20 (three peptides). Therefore, 20 peptide pools were used for this epitope mapping.

Cell separation and culture conditions

Blood (100 ml) from each subject was collected in preservative-free heparin and separated by standard techniques using Lymphoprep (Nycomed, Oslo, Norway). Mononuclear cells were harvested and washed twice in RPMI 1640 (GIBCO, Grand Island, NY) and then resuspended in complete medium (RPMI supplemented with 10% human AB-serum; ICN Flow, High Wycombe, UK), 2 mM L-glutamine and 20 µg/ml gentamicin (GIBCO). Cells were cultured at a concentration of 2×10^5 /well in 0.2 ml of complete medium in U-bottomed 96-well microtitre plates. Since the precursor frequency of T cells specific to any one peptide is very low, 12 replicate cultures were used for each of the 20 peptide pools. Twenty pools were added at a final concentration of 1 µg/ml of each peptide. Purified *Lol p I* or *L. perenne* extract (LPE) were added at a concentration of 1 or 10 µg/ml as a positive control. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. After 6 days, 0.4 µCi/well methyl-³H-TdR (Amersham, Aylesbury, UK) was added and 6 h later the cells were harvested onto Titertek glass-fibre paper (ICN Flow). ³H-thymidine incorporation into DNA was measured by liquid scintillation counting (Minaxi Tri-Carb 4000 Series, Packard) and taken as an index of the PBMC proliferative response to the purified *Lol p I*, LPE or peptide pools.

PBMC responses to overlapping peptides

Twenty pools of peptides were screened, each pool comprising six overlapping peptides (with the exceptions noted above) with successive peptides differing in only two amino acids in their starting (N-terminal) residue. Each pool was tested in at least 12

replicate wells of PBMC. The thymidine incorporation of each replicate was measured.

Data analysis

Most of the peptides do not lead to thymidine incorporation and can therefore be considered as background counts. In addition, 48 wells per experiment were used as pure background counts, i.e. wells without any added peptides. For each pool or peptide, 12–24 replicate wells were studied. We have taken the mean of all wells (336) in a particular experiment on a particular patient, and any well with counts above the mean ± 3 s.d. is considered as positive with a confidence limit of 99.99%. For each experimental group in each patient, the percentage of positive wells was plotted against the pool number.

Decoding of the stimulatory pool

Following the identification of a stimulatory pool, the 'decoding' was carried out by culturing each single peptide of that pool with PBMC of the patient by the methods described above.

Dose response curve

Following the identification of the stimulatory pool and single peptides, a dose response study was carried out with different concentrations of the positive pool and its single peptide, a negative pool, and Lol p I.

Study of overlapping peptides in vivo

Twenty peptide pools were studied *in vivo* by intradermal skin testing of four atopics and four non-atopics. Eight pools were tested on each occasion using the volar surface of the forearms, the skin test response being studied at various times from 20 min to 72 h. The dose of peptide injected was varied from 2 μ g/ml to 25 μ g/ml. The skin response was also used to decode the positive pool. All skin tests were carried out before the pollen season. Delayed skin test responses were measured at 24, 48 and 72 h.

RESULTS

Preparation and characterization of Lol p I

Purification of rye grass extract by FPLC gives two peaks (peaks 1 and 2) (Fig. 1). Electrophoresis of a *L. perenne* extract under reducing conditions followed by protein staining showed that the mol. wt of peak 1 was approximately 31 kD and its location was equivalent to the highly purified Lol p I kindly supplied by Professor David Marsh (Johns Hopkins University, Baltimore, MD). The other peak was identified as a mixture of Lol p II and Lol p III with a mol. wt of 11 kD (data not shown).

IgE binding activity of purified Lol p I

To study the IgE binding activity of Lol p I purified by FPLC, skin testing was performed with the purified Lol p I and LPE on the forearm of grass pollen-sensitive and non-atopic subjects. Purified Lol p I showed parallel skin reactivity compared with LPE (Table 1), as did the IgE binding activity *in vitro* as determined by ELISA (data not shown).

PBMC responses to Lol p I and LPE

Human PBMC proliferative responses to the major purified fraction of *L. perenne* (Lol p I) and LPE are shown in Fig. 2. Atopics responded similarly to both Lol p I and LPE. Non-atopics did not respond significantly to Lol p I, but did show

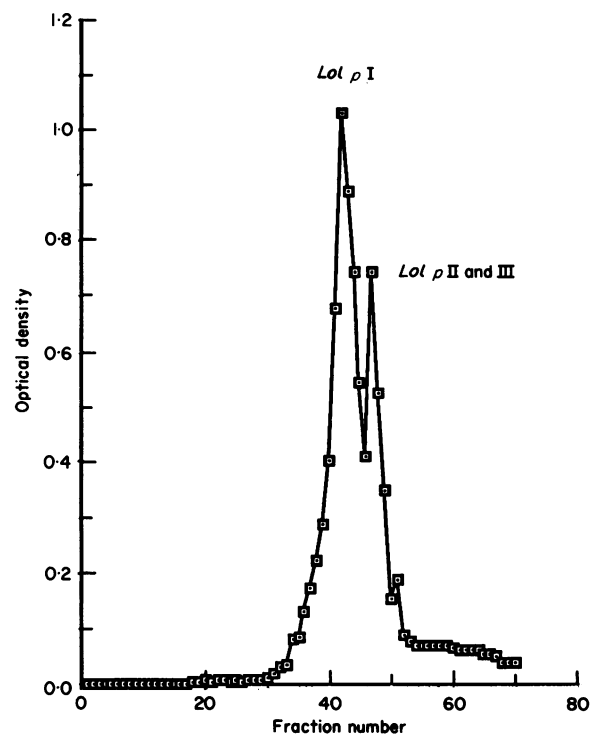


Fig. 1. Fractions of *Lolium perenne* extract (0.3 ml) were separated by fast performance liquid chromatography (FPLC) on a gel filtration column (Superose 12). Optical density of each fraction was measured at 280 nm.

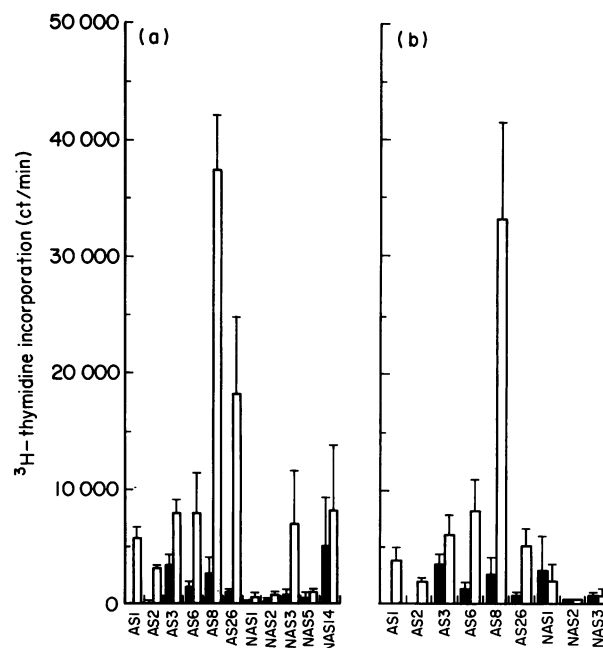


Fig. 2. In peripheral blood mononuclear cell (PBMC) responses, most atopics showed significant and parallel stimulation to *Lolium perenne* extract (LPE) (a) and Lol p I (b), whereas the non-atopics showed little or no response. However, thymidine incorporation above background was seen in some non-atopic cells incubated with LPE but not in response to Lol p I. The error bars refer to the s.d. of the 12 replicate wells. ■, Background; □, LPE (a), Lol p I (b).

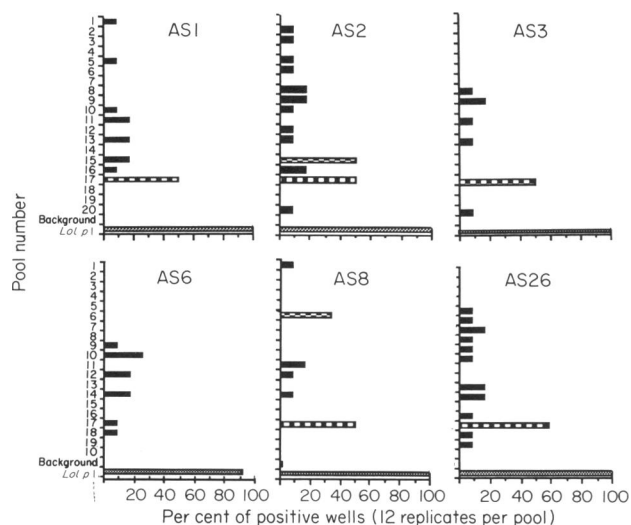


Fig. 3. Percentage of positive wells of the peptide-specific T cells in peripheral blood mononuclear cells (PBMC) in six rye grass-sensitive patients using 20 peptide pools spanning the entire length of the iso-allergens clone1A of *Lol p I*. Each pool was composed of six overlapping 12mer peptides offset by two residues, and thus spans 22 residues of the protein sequence. In five out of the six patients, pool-17 was the dominant epitope.

some stimulation with LPE. PBMC from all subjects showed comparable responses to the non-specific T cell mitogen concanavalin A (Con A) and a specific T cell antigen PPD (data not shown).

PBMC responses to synthetic peptides

The presence of T cell epitopes in the 20 pools of peptides was investigated by *in vitro* proliferation tests on PBMC of the six rye grass-sensitive patients and the five non-atopic control subjects. The main T cell stimulation was obtained with pool-17 in five out of six atopics, although one atopic (AS2) did show stimulation to pool-15 and AS8 showed stimulation to pool-6 as well as pool-17 (Fig. 3). Non-atopic subjects did not respond to any of the 20 peptide pools.

Decoding of the stimulatory pool

Following the identification of pool-17 as the most common stimulatory pool, 'decoding' was carried out on PBMC of subject AS3 (Fig. 4). This shows that peptide 3 and 4 of pool-17 are the stimulatory peptides *in vitro*. The same results were obtained with two other atopics, AS8 and AS26, and three successive samples of PBMC from subject AS3. In the series of overlapping 12mer peptides, those with amino-terminal residue 193–206 (Trp-Gly-Ala-Val-Trp-Arg-Ile-Asp-Thr-Pro-Asp-Lys-Leu-Thr) stimulated the PBMC of rye grass-sensitive patients to the greatest extent. This sequence therefore contains the major T cell epitope of the iso-allergen clone1A of *Lol p I* of rye grass.

Dose response curve

Following the identification of the stimulatory pool, a dose response curve was carried out with the positive pool (pool-17) and its single most active peptide (peptide 4), a negative pool (pool-8), and *Lol p I*. Results are shown in Fig. 5. Even at high concentrations the negative pool did not lead to any stimu-

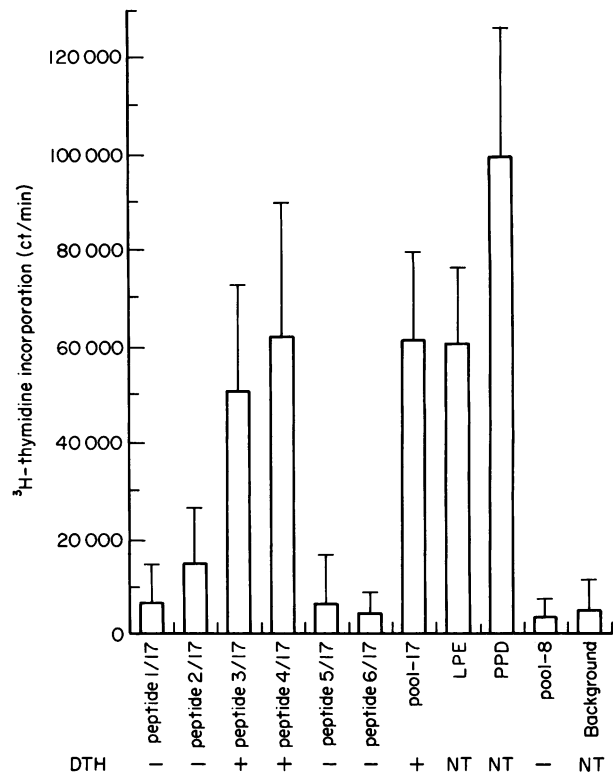


Fig. 4. Decoding of the stimulatory pool (pool-17) in an atopic patient. Peripheral blood mononuclear cells (PBMC) were cultured with six peptides of pool-17 separately at a final concentration of 2 µg/ml of each peptide. Purified protein derivative (PPD), pool-17, *Lolium perenne* extract (LPE), pool-8 and cells alone were cultured as control. These peptides were tested *in vivo* by intradermal injection of each peptide separately. The individual peptides that induced thymidine incorporation (peptides 3 and 4) also led to DTH when injected intradermally. NT, Not tested.

tion, whilst the positive pool (pool-17) and the single peptide gave a dose response curve similar to that of *Lol p I*.

In vivo study of allergen peptides

Only one pool (pool-17) out of the 20 peptide pools tested gave a typical DTH response on skin testing with a delayed time course, which peaked at 24 h and was still detectable at 48 h. The diameter of the indurated area was 9 mm at 24 h. The test area where positive was firm, with slight overlaying erythema. One out of the four atopic patients tested showed this response, whilst non-atopics did not show any reaction.

Dose response curve can be seen by using different concentrations of pool-17. Using 12.5 µg/ml and 25 µg/ml of pool-17 showed 9 mm and 13 mm induration, respectively.

Decoding the positive pool by testing single peptides *in vivo*, we were able to show DTH responses to peptides 3 and 4 of the positive pool (pool-17) (Fig. 4), an exact parallel of the *in vitro* results.

DISCUSSION

In this study we have characterized the immunodominant T cell epitope of the iso-allergenic clone1A of *Lol p I*, the major fraction of the most common outdoor grass pollen (*L. perenne*). The response has been defined not only *in vitro* but also *in vivo* in

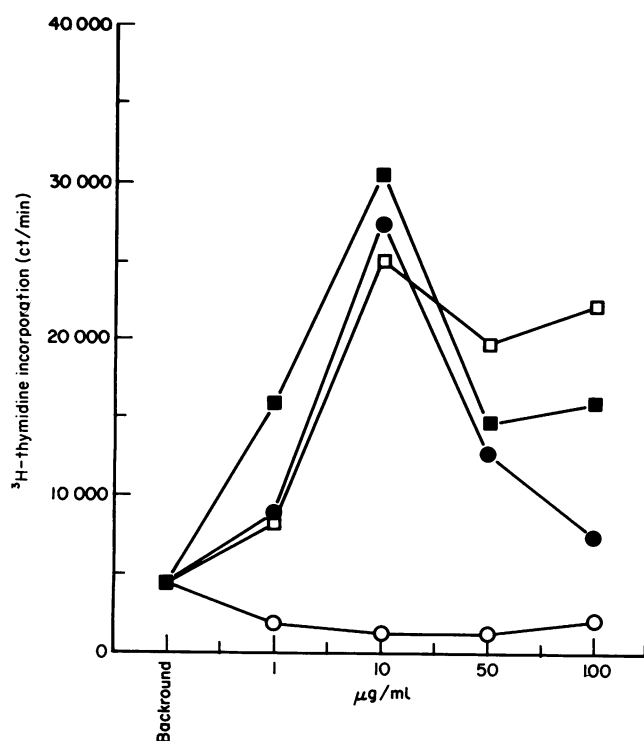


Fig. 5. Dose response curve to Lol p I, positive pool (pool-17, □) and its single peptide (peptide 4, ■) and negative pool (pool-8, ○) in an atopic patient (AS3). The purified major fraction (Lol p I, ●), pool-17 and the specific peptide from pool-17 (peptide 4) all stimulate peripheral blood mononuclear cells (PBMC) with the same dose response curve, whereas the negative pool (pool-8) has no effect even at high concentration.

one of four atopic subjects tested by induction of DTH responses in the skin. The response was a typical DTH which peaked at 24 h, and was still appreciable at 48 h. Non-atopics did not show a DTH response to any of the peptide pools, even at high peptide dose (50 µg/ml). However, some of the non-atopics did show T cell proliferation to the unpurified whole pollen extract.

This T cell epitope, to which most of our patients react *in vitro*, can be considered the major stimulating peptide in this group of allergic subjects. By using 12mer peptides overlapping by 10, the site of the T cell epitope has been located with high precision. There are thus 10 amino acids common to the two most stimulatory peptides, and up to 14 amino acids may be involved, of which seven out of the 14 are hydrophobic. Our T cell epitope with 14 amino acids (Trp-Gly-Ala-Val-Trp-Arg-Ile-Asp-Thr-Pro-Asp-Lys-Leu-Thr) confirms the study of Perez *et al.* [8], who used T cell clones from atopic subjects and 20mer peptides overlapping by 10. The sequence found in that study was as follows: (Glu-Ser-Trp-Gly-Ala-Val-Trp-Arg-Ile-Asp-Thr-Pro-Asp-Lys-Leu-Thr-Gly-Pro-Phe-Thr). We feel, however, that PBMC better reflect the *in vivo* situation. The epitope that we describe has six amino acids less than the one described by Perez. However, in that study, they used 20mer peptides overlapping by 10, which gives a lower degree of accuracy for the identification of the exact epitope. We have also shown that non-atopics did not respond to either the peptide pools or Lol p I, and three out of the five did not react to LPE. Our data show that the precursor frequency in non-atopics is low, insufficient to be measured by stimulation with allergen peptides.

Although some groups have also shown no significant response in non-atopic controls [17], other groups have shown some lymphocyte activation by allergen extracts in non-atopics [18,19]. The conflict between our data and those of the other groups may be explained by the use of different *in vitro* systems. First, we have used a purified antigen and short synthetic peptides covering the whole sequence of major fraction (Lol p I), whereas other groups have used a crude whole pollen extract [18]. Second, we have used PBMC whereas others have used T cell lines or T cell clones [20,21]. Dominant immune responses to antigen can be seen in bulk cultures, but these may not necessarily reflect the presence of large numbers of precursors, since the selection processes *in vitro* may favour the expansion of cell types which do not predominate *in vivo*. Thus, a T cell clone which reflects a highly enriched lymphocyte population cannot represent the whole population of allergen-specific T cells that exists in peripheral blood. Our system eliminates this source of bias in selection of the lymphocyte population. By incubating PBMC with different concentrations of Lol p I and allergen peptide in 12, 24 or even in some cases 48 replicates we did not find any response in non-atopics to peptide pools. However, five out of the six atopic subjects in our study did show a specific response to pool-17.

At a clinical level we view the non-atopic as an effective 'non-responder' to allergen *in vivo*, even though there may be a small number of antigen-specific lymphocytes circulating which can perhaps be shown by generating T cell clones [20]. However, Th1-like cells in normal subjects may be activated by common allergens (e.g. house dust mite), but because of the secreted lymphokine profile [21] and low precursor frequency, do not give rise to the production of IgE. Indeed, an on-going Th1 response to allergens associated with the production of interferon-gamma (IFN-γ) may be protective in the sense of inhibiting a potential IL-4-induced IgE production [22–24].

In the atopic we do not know which subset of T cell is responsible for DTH reactions. In the mouse, Cher & Mosmann [25] have shown that the Th1 subset is responsible for DTH, although recent studies in atopic dermatitis in man [26] have suggested that Th2-like cells can also be implicated. A recent study has shown that cells infiltrating the site of the 24-h late phase reaction, which is subsequent to an IgE-mediated reaction, i.e. not a classical DTH response, have a similar cytokine profile to the Th2 subset [27].

Clinical hyposensitization for hay fever using an incremental regime of injection of whole extract of allergens is not curative, although symptoms are ameliorated. However, whole extracts contain an IgE binding site which can result in anaphylactic shock and even death [1]. Although pharmacological treatment can relieve mild symptoms, there is as yet no immunological 'cure' for the severe cases, especially those with asthma.

One approach to overcome these problems has been the development of modified allergens for use in vaccines which might first be more effective, and second, much safer.

As T cells have a crucial role in the control of IgE, any manipulation which gives rise to specific unresponsiveness or anergy in the specific T cell population may lead to a reduction in specific IgE and help the patient, such as allergen-derived peptides [28], non-stimulatory unrelated peptide [29], and anti-CD4 [30–32].

Individuals in a heterogeneous population may respond to different unique T cell epitopes on the same protein [33], so that

a single T cell epitope might not suffice for effective allergen immunotherapy in an outbred human population. However, the rye grass T cell epitope to which most of our patients react, could be considered an immunodominant peptide for potential treatment of this group of rye grass-sensitive atopic subjects. A clear clinical advantage in using a vaccine containing the T cell epitope would be the absence of anaphylactic reactions through triggering IgE on mast cells. There is the possibility that a specific T cell epitope vaccine might provide help for Th2 cells and actually boost IgE production. This is a consideration that must be taken into account. Regulation of the immune response to pollen allergen with an enzyme digest of pollen has already been shown to be successful in both animal [34] and man [35]. The T cell epitope found here did not show any immediate hypersensitivity reactions (i.e. wheal and flare) on skin testing rye grass-sensitive patients. This suggests that a similar approach with the immunodominant epitope that we describe might be effective clinically.

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