

Recognition of T Cell Epitopes and Lymphokine Secretion by Rye Grass Allergen *Lolium perenne* I-Specific Human T Cell Clones¹

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T cell lines (TCL) and CD4⁺ T cell clones (TCC) with specificity for the rye grass allergen *Lolium perenne* (*Lol p*) I were isolated from the blood of nine donors, six having active atopic disease, two being in remission, and one having IgE anti-*Lol p* I Abs but not atopic disease. The T cell epitopes of *Lol p* I were determined by TCLs and TCCs reactivity with 23 overlapping, 20 amino acid-long peptides spanning the entire length of the 230 amino acid-long allergen. In addition, the Th subsets (Th1, Th2, Th0, Thp) were determined by measuring IL-2, IFN- γ , and IL-4 in the supernatants of TCC activated with *Lol p* I and irradiated APC. TCC from individuals from which a large panel of clones were obtained from 10⁵ PBMC initial cultures recognized multiple peptides (5–9) and of 23 overlapping peptides a total of 16 were recognized by at least one TCC from one of the patients. These 16 peptides were derived from all areas of the *Lol p* I molecule, indicating the ability of human Th cells to recognize many peptide epitopes on *Lol p* I. Although no clear cut immunodominant peptides were detected, T cell clones of 50% of the patients reacted with peptide 191–210. There was no correlation between peptide epitope reactivity and lymphokine secretion pattern of the TCC. Of 12 TCC obtained from six patients with active atopic disease, four (33%) were of Th1, five (42%) of Th2, one (8%) of Thp, and two (17%) of Th0 type. Of 14 TCCs isolated from three atopic donors in remission, five (36%) were of Th1, three (21%) of Th2, four (29%) of Thp, and two (14%) of Th0 type. The data demonstrate that T cells from rye grass pollen allergic patients can recognize multiple peptide epitopes on *Lol p* I scattered over the entire molecule. No correlation existed between epitope reactivity and lymphokine secretion pattern of the TCC. *Journal of Immunology*, 1994, 152: 4706.

The Ag receptor of Th cells recognizes a relatively short peptide epitope of approximately 13 to 17 amino acids in length that is bound to MHC class II molecules on APC (1). The Th cells become activated to proliferate and secrete lymphokines after interaction of the TCR with this MHC class II-peptide complex together with a second signal provided by the APC (2). In contrast, Th cells are rendered unresponsive (anergic, tolerant) if they react with the peptide epitope in the absence of the

second T cell activation signal (3). Therefore, immune responses to undesirable Ags, such as autoantigens and allergens, might be prevented by injecting patients with the T cell reactive peptides, a procedure that was shown to down-regulate the Ab responses to the cat allergen *Fel d* I in mice (4).

IgE Abs to allergens are the predominant and most likely only isotype of Abs responsible for inducing allergic diseases in humans (5). IgE Ab formation is highly T cell dependent (6, 7) and could therefore be targeted for desensitization by injection of synthetic peptide T cell epitopes of allergens because the peptides do not bind to already formed IgE Abs and therefore would not induce release of mediators from basophils and mast cells (8–10).

Recently, the primary structure of several major allergens has been determined by DNA sequencing methodology (11–17). However, relatively little is known regarding the number of epitopes that are recognized by allergen-specific Th cells from atopic patients. By using in vitro

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generated T cell lines specific for the bee venom allergen phospholipase A₂ from one atopic patient, only two peptide epitopes were detected (18). In contrast, TCC³ isolated from six birch pollen *Bet v* I-sensitive (8) patients reacted with at least seven peptide epitopes scattered over the entire protein. Similarly, T cell lines isolated from house dust mite *Der p* II-sensitive patients (19, 20) reacted with more than 10 different peptides from all areas of the molecule. However, in one study of *Der p* II-specific lines, two areas of 40 amino acids in length were reported to contain immunodominant T cell epitopes (20). Therefore, rendering T cells anergic with peptide epitopes may provide a future therapy for allergies, especially if an allergen contains only a few immunodominant epitopes.

The purpose of this investigation was to determine the peptide epitope reactivity of Th cells from patients allergic to the major Ag *Lol p* I of rye grass pollen. For this purpose *Lol p* I-specific T cell lines and clones were isolated from nine rye grass pollen-sensitive individuals and tested for their reactivity with 23 overlapping peptides of 20 amino acid length spanning the entire length of the 230 amino acid molecule (14). In addition, the IL-2, IFN- γ , and IL-4 secretion of TCC was quantitated to determine whether an association exists between the T cell epitope reactivity and lymphokine secretion pattern.

Materials and Methods

Patients

Heparinized venous blood was obtained from nine donors who had either active allergic disease or were in remission. Detailed clinical data of the patients are described in the *Results* section. All donors gave informed consent.

Lol p I and synthetic peptides

Lol p I was obtained from the NIAID Research Resources or was isolated from rye grass pollen (Greer Laboratories, Lenoir, NC) as previously described (21). Goat anti-sera to *Lol p* I and whole rye grass extract for antigenic analyses during purification were kindly provided by Dr. David Marsh (Johns Hopkins University, Baltimore, MD). The *Lol p* I preparations showed a single band when analyzed by PAGE and one precipitin line by Ouchterlony assays using the anti-whole pollen antiserum. The synthesis of a set of 23 overlapping peptides, each 20 amino acids long, encompassing the entire *Lol p* I molecule was previously described (14). Each successive peptide overlapped the preceding peptide by 10 residues.

RAST

For direct RAST assays, 96-well microtiter plates were coated with *Lol p* I at 5 μ g/ml BBS 0.15 M NaCl, pH 8.6. After 1 h at room temperature, the plates were washed and the nonspecific protein binding sites blocked by addition of 5% nonfat milk in BBS for at least 1 h. Serial twofold dilutions of the sera beginning at 1:10 for IgE and 1:1000 for IgG were then added overnight. After washing the plate, ¹²⁵I-radiolabeled polyclonal purified goat anti-human ϵ - or γ -chain-specific Abs prepared in our laboratory were added. Each well received approximately 20 ng Abs emitting 500,000 cpm. After 4 h of incubation, the plates were washed and the radioactivity of the wells counted and compared with a standard serum. IgE anti-*Lol p* I Abs were quantitated by a reverse RAST

assay (22). For this assay, plates were coated with a mixture of two monoclonal anti- ϵ chain Abs, kindly provided by Dr. A. Saxon (UCLA, Los Angeles, CA) to capture the IgE. After washing and blocking nonspecific sites, 100 μ l of serial dilutions of the patient's sera were added overnight. ¹²⁵I-radiolabeled *Lol p* I, 500,000 cpm per well, was then added for 4 h, after which the plates were washed and counted. The serum of patient S149 who had the highest IgE anti-*Lol p* I titer was used as the standard and arbitrarily assigned 1000 U anti-*Lol p* I/ml. The U/ml of anti-*Lol p* I Abs in the other sera were calculated on the basis of the standard curve obtained with serum of patient S149.

Isolation of TCC

Two procedures were used to isolate TCC. First, the clones were isolated according to the method described by Parronchi et al. (23). Briefly, 1×10^6 PBMCs were incubated with 10 μ g *Lol p* I/ml in 2 ml medium in 24-well plates for 5 days. The medium was RPMI 1640 supplemented with 5% autologous or AB serum, antibiotics, glutamine, sodium pyruvate, and nonessential amino acids. Five days after initiating the culture, 20 ng IL-2/ml was added to the culture. The rIL-2 was kindly provided by Dr. H.P. Kocher (Sandoz, Basel, Switzerland). On day 11, 200 μ l of the cell culture was collected, 1 μ Ci [³H]thymidine added for 18 h, and the cell proliferation determined the next day by ³H uptake. Cells from wells showing two to five times more proliferation than control cultures that did not receive *Lol p* I were centrifuged over Ficoll-Hypaque and the T cell blasts were then cloned. For this purpose 0.33 and 1 cell/well were seeded in 96-well plates with irradiated autologous PBMC (10^5 /well), 20 ng IL-2, and 1 μ g PHA/ml (purified PHA, Murex Diagnostics, Greenville, NC).

The wells were observed for cell growth and cells from plates with wells having <33% positive growth were expanded into 24-well plates by addition of the irradiated PBMCs and IL-2. On day 35, the clones were screened for *Lol p* I-specific proliferation and lymphokine secretion and subcloned as necessary. Once the clones were isolated 10% FCS was used in the medium instead of human serum. The specific TCCs were expanded by incubation of 2×10^5 T cells with 2×10^6 irradiated APC, PHA, and IL-2. The cells were collected and frozen in liquid nitrogen. During the course of performing these experiments it was learned that most of the TCC derived from one original well containing 10^6 PBMC/well from some patients had an identical peptide specificity and similar lymphokine secretion pattern, suggesting that they may have been derived from the same precursor cell. Therefore, a second method was used to isolate TCC. This method was similar to the first except for seeding only 10^5 PBMC/per well with 10 μ g *Lol p* I/ml in 96-well plates.

After day 12, the cells were divided into three 96-well plates. In two plates, *Lol p* I and irradiated APCs were added, whereas the third plate received only APCs. Fresh medium with IL-2 was then added every third day for 10 days. One microcurie [³H]thymidine was added per well to one of the plates containing APC and *Lol p* I and to the control plate without *Lol p* I and the cell proliferation determined 18 h later. This allowed for the identification of wells that show *Lol p* I-specific growth while also eliminating cultures containing autoreactive T cells. The cells from lines showing *Lol p* I specific proliferation were then cloned at 0.33 and 1 cell/well. The resulting clones from plates showing <33% clonal growth were retested for *Lol p* I specificity. TCL and TCC were tested for reactivity with synthetic peptides as previously described (14). The TCC clones were analyzed for CD4 or CD8 type by using monoclonal OKT4 and OKT8 Abs (Ortho Pharmaceutical, Raritan, NJ) followed by fluoresceinated goat anti-mouse IgG.

T cell proliferation assay

In 96-well round bottom plates, 2×10^4 T cells and 8×10^4 5000 rads irradiated autologous PBMC and 5 to 10 μ g/ml *Lol p* I or synthetic peptide were cultured for 2 days before 1 μ Ci [³H]thymidine was added to each well. Thymidine uptake was determined on day 3. In some experiments, 50 μ l of supernatant used for lymphokine determination was collected on day 2 of culture and replaced with an equivalent volume of medium containing 1 μ Ci [³H]thymidine.

IFN- γ , IL-4, and IL-2 determinations

The supernatants of activated TCC were collected 48 h after *Lol p* I stimulation and stored frozen at -20°C before analyses for lymphokine

³ Abbreviations used in this paper: TCC, T cell clone; *Lol p*, *Lolium perenne*; BBS, borate buffered saline; RAST, radioallergosorbent test.

Table I. Clinical data and anti-Lol p I Abs of donors providing blood for the isolation of Lol p I-specific TCC

Patient	Age/Sex	Atopic Disease ^a	Rye Grass Skin Test (Dilution)	Total IgE (ng/ml)	RAST		HLA-DR Haplotype
					IgE (U/ml)	IgG (cpm)	
S149	18/F	AR	4+(10 ⁻⁵)	474	1000	2596	4
UC1	32/M	AR ^b	2+(10 ⁻⁵)	668	956	5410	1,7
UC2	28/F	A	3+(10 ⁻⁶)	526	57	2867	1,w11
S176	24/F	A,AR	3+(10 ⁻⁵)	91	28	1161	2
S207	28/F	A,AR	2+(10 ⁻⁵)	258	16	145	5,w13
S178	27/M	A,AR	3+(10 ⁻⁶)	128	119	479	3,7
S158	41/F	AR	4+(10 ⁻⁵)	348	38	2816	2,7
UC3	59/M	A,AD ^c	neg(10 ⁻³)	28	0	6221	4,w11
S132	36/M	None	4+(10 ⁻³)	423	50	483	1,3

^a A, asthma; AR, allergic rhinitis; AD, atopic dermatitis.

^b In remission, no symptoms for 8 yr.

^c AD, A in childhood only.

secretion by bioimmunoassay or RIA. The RIAs were performed as previously described (24) except that mAbs to IFN- γ or IL-4 were used. For IFN- γ determinations, the plates were coated with mAb no. 69 kindly provided by Dr. H. Gallati (Hoffman-LaRoche, Basel, Switzerland). The mAb K3.53.3H2 to IFN- γ , kindly provided by Dr. R. Schreiber (St. Louis, MO), was used as radiolabeled second Ab. Two mAbs, 9G7 and 11E6, recognizing two different epitopes on IL-4 were prepared in our laboratory using rIL-4 provided by Dr. H. P. Kocher (Sandoz). The sensitivity of the two RIAs was approximately 30 pg/ml. IL-2 was determined by a bioassay using CTLL cells.

Results

Patient population

The clinical data of the nine rye grass pollen-sensitive humans that served for the isolation of TCC are shown in Table I. TCCs were isolated from six patients with active allergic rhinitis and/or asthma at the time blood was obtained for T cell cloning. The allergic disease interfered with normal lifestyle in these patients, despite symptomatic therapy. For this reason five of six received immunotherapy after blood donation for the described experiments. One patient was in remission (UC1) and one (UC3) had had atopic disease in childhood but no allergic disease since the age of 25 yr. UC3 was Lol p I skin test and IgE RAST negative but had a high titer of IgG anti-Lol p I Abs. One donor (S132) had both IgE and IgG anti-Lol p I Abs but never had clinically apparent allergic disease. Except for UC3, all had positive skin tests to grass pollen extract. None of the patients had received oral corticosteroids or grass pollen immunotherapy before blood donation. There was no dominant HLA-DR haplotype among the nine Lol p I-sensitive donors.

Peptide epitope reactivity of TCC

Initially, 1×10^6 PBMC in 24-well plates were stimulated with purified Lol p I and IL-2 for 12 days and the Lol p I-induced cell proliferation determined. Cells from wells demonstrating a Lol p I-specific proliferation (T cell lines) were then used for cell cloning. In some experiments, the T cell lines were studied for peptide reactivity. As shown in Table II for seven T cell lines from patient UC1, four T

Table II. Peptide reactivity of 7 Lol p I-positive T cell lines derived from 10⁵ PBMC from patient UC1

Line	Reactive Peptides
A1	121–140
A3	151–170
B6	None
B11	None
D4	1–20, 50–70, 111–130, 131–150
F6	None
G7	181–200

cell lines proliferated to peptide. Three lines showed reactivity with one peptide, each being derived from a different location on the Lol p I molecule. A fourth line reacted with four peptides, two of them located in the area of amino acids 111 to 150. To study the ability of Th cells of Lol p I allergic patients to recognize different T cell epitopes on Lol p I, TCCs were generated from specific TCL. Many TCC isolated from a primary culture of 10⁶ PBMC had the same peptide specificity and lymphokine secretion pattern suggesting that the TCC may have been derived from the same precursor cell. Therefore, T cell lines were subsequently generated from 10⁵ PBMC to avoid studying TCC that may have been derived from the same precursor cells.

As shown in Figure 1, 16 of 23 synthetic peptides were recognized by at least one TCC from eight rye grass-sensitive donors. These peptides were located throughout the entire Lol p I molecule. The few clones obtained from donor UC2 did not proliferate to any of the peptides. A large panel of TCC was isolated from patients S132, S149, S158, and S178. TCC from each of these four individuals recognized between five and nine different overlapping peptides (Fig. 1). Twenty-two percent of all the clones tested and every clone of patient UC2 failed to react with any of the peptides, despite a strong proliferative response to Lol p I.

FIGURE 1. Schematic representation of synthetic peptide epitope reactivity of TCC obtained from eight *Lol p I*-sensitive donors. If a TCC reacted with two overlapping peptides, the peptide that induced the stronger proliferation is shown in the figure.

Peptide Number	Amino Acid Sequence	Patient							
		S132	S149	S158	S176	S178	S207	UC1	UC3
1	1-20								
2	11-30								
3	21-40								
4	31-50								
5	41-60								
6	51-70								
7	61-80								
8	71-90								
9	81-100								
10	91-110								
11	101-120								
12	111-130								
13	121-140								
14	131-150								
15	141-160								
16	151-170								
17	161-180								
18	171-190								
19	181-200								
20	191-210								
21	201-220								
22	211-230								
23	221-240								

Lymphokine secretion by TCC

To determine whether a correlation existed between peptide reactivity and lymphokine secretion, 12 clones from six patients with active disease and 14 clones from the donors in remission were analyzed for IL-2, IFN- γ , and IL-4 secretion. For this purpose the TCCs were activated with *Lol p I* and APCs. The T cell subsets were recorded as Th1 if there was IFN- γ but no detectable IL-4 secretion or if the IFN- γ /IL-4 secretion ratio was >10 . Similarly, cells secreting IL-4 and no or little IFN- γ were classified as Th2 cells. Cells that secreted approximately equal amounts of IFN- γ and IL-4 were termed Th0 cells. TCC that strongly proliferated to *Lol p I* but secreted only IL-2 and no detectable IFN- γ or IL-4 were designated Thp (precursor) (25). As has previously been shown, IL-2 secretion does not parallel IFN- γ secretion by human TCC (26) in contrast to murine TCC (27). Therefore, only IFN- γ and IL-4 secretion were used for the Th subset classification. As shown in Table III, of the 12 TCC isolated from the six patients with active disease at the time of blood donation, four (33%) were of Th1, five (42%) of Th2, one (8%) of Thp, and two (17%) of Th0 type. Of 14 TCC isolated from the two atopic donors in remission or from the clinically nonatopic human, five (36%) were of Th1, three (21%) of Th2, four (29%) of Thp, and two (14%) of Th0 type. There was no apparent correlation between lymphokine secretion pattern and peptide epitope reactivity. Six TCCs were expanded with PHA, IL-2, and irradiated PBMC over a 2-yr period and retested for their lymphokine secretion pattern. Although the quantity of IFN- γ or IL-4 secretion varied

with different cell batches, the Th1 or Th2 phenotype of the different TCC did not change (data not shown).

Discussion

These data demonstrate that Th cells from rye grass pollen-sensitive atopic patients have the ability to recognize multiple peptide epitopes that are located along the entire *Lol p I* molecule. Isolated TCC from different patients reacted with 16 of 23 peptides of 20 amino acids in length, indicating that the T cells could recognize at least 16 epitopes. This number is most likely higher because peptide epitopes are usually of approximately 12 amino acid length and a 20 amino acid peptide could contain two epitopes. Testing with shorter peptides that have smaller overlaps would be necessary to identify all T cell epitopes that can be recognized by human Th cells. Furthermore, many TCC that proliferated strongly to *Lol p I* did not react with any of the synthetic peptides, perhaps because the TCC recognize peptides from different *Lol p I* isoforms and our synthetic peptides represented only one *Lol p I* isoform (14) or because the synthetic peptides were processed by the APCs differently from the native *Lol p I* or were digested to smaller peptides that no longer form structures recognized by the T cells. Taken together, the data show a large repertoire of human Th cells able to recognize many *Lol p I* T cell epitopes.

Our data are similar to those of recent reports regarding the T cell epitopes on birch pollen *Bet v I* (8) and house dust mite allergen *Der p II* (19, 20). Eleven of 13 TCCs isolated from six birch pollen allergic patients reacted with

Table III. Summary of peptide reactivity and lymphokine secretion of 26 TCC obtained from eight donors

	No. Clone	Reactive Peptide	IL-2 (U/ml)	IFN- γ (pg/ml)	IL-4 (pg/ml)	Th Subtype
Patients with active atopic disease						
S149	3.14	151-170	640	<100	2,262	Th2
	8.20	31-50	40	<100	1,078	Th2
	43.62	151-170	<20	<100	568	Th2
	141.02	101-120	320	1,405	<60	Th1
S176	214.6	191-210	320	<100	559	Th2
S207	17.08	71-90	40	<100	210	Th2
	47.03	21-40	320	515	830	Th0
	55.18	171-190	<20	585	<60	Th1
	55.13	121-140	320	950	507	Th0
S178	12.35	91-110	<20	<100	<60	Thp
	12.41	141-160	2,560	2,410	<60	Th1
	A.10	Neg	640	2,818	233	Th1
Patients in remission and healthy donor						
UC1	5.13	Neg	40	28,963	1,172	Th1
	5.17	Neg	<20	743	7,063	Th2
	B6.5	Neg	<20	794	601	Th0
	B6.6	41-60	<20	<100	<60	Thp
	H2.7	91-110	<20	>20,000	<60	Th1
	G2.1	181-200	<20	975	3,303	Th2
	H5	190-210	<20	3,226	<60	Th1
UC3	B21F7	Neg	<20	1,263	<60	Th1
S132	6.40	221-240	<20	<100	845	Th2
	105.18	171-190	<20	<100	<60	Thp
	109.23	221-240	<20	<100	<60	Thp
	136.10	91-110	160	245	224	Th0
	166.1	1-20	<20	<100	<60	Thp
	166.18	171-190	<20	250	<60	Th1

seven distinct overlapping synthetic peptides that were scattered over the entire 159 amino acid-long allergen (8). Similarly, T cell lines from five house dust mite-sensitive patients reacted with at least 10 different peptide epitopes and each donor showed a different pattern of reactivity (9). These data indicate that in atopic individuals the Th cells specific for these grass and tree pollens and to house dust mite allergens can recognize multiple peptide epitopes on these allergens.

Although these experiments demonstrate that Th cells can recognize many peptide epitopes on pollen allergens, the question remains whether some of these epitopes are dominant in vivo. In some patients such as S176, UC2, and in a previously reported patient (14) the TCCs reacted with only one or two peptides suggesting that these peptides were dominant in these patients. Interestingly, at least one TCC from each of these three patients recognized peptide 191 to 210 and this may well be an immunodominant peptide in these patients. However, TCC from only 50% of the patients reacted with peptide 191 to 210 suggesting that it is not a dominant peptide in all patients with rye grass allergy. The method used in our studies to isolate TCC from T cell lines obtained from 10^5 PBMC favored detection of the entire repertoire of T cells able to recognize a Lol p I peptide epitope. Whether in vivo the majority of allergen specific T cells are directed to only one or two immunodominant peptides is unknown. Testing of T cell lines from patient UC1 obtained after one cycle of Lol p I stimulation showed that T cells in four of seven lines rec-

ognized seven different epitopes suggesting that in this patient the Lol p I Th cells are not restricted to one or two dominant peptides.

Dhillon et al. (18) detected only two peptide epitopes on the major bee venom allergen phospholipase A₂ by testing T cell lines from one patient. Two major immunodominant peptides were reported for the cat allergen *Fel d I* (4). Whether examination of many TCCs rather than T cell lines would detect additional T cell epitopes on these allergens remains to be determined. It is also possible that various allergens differ with respect to expression of multiple T cell epitopes.

As has been shown by us in this study and by others, IL-2 secretion is not typical for all human Th1 cells (8, 23, 26) in contrast to murine Th1 cells (27). Consequently, we designated cells that secrete IFN- γ and very little or no IL-4 as Th1 cells. Analogously, IL-4-secreting cells or cells showing a high ratio of IL-4:IFN- γ secretion were designated as Th2 cells. Conceivably, individual peptide epitopes could elicit the differentiation of either Th1 or Th2 cells to this epitope. If certain peptides from allergens induce the differentiation of Th2 cells, then induction of T cell anergy to such peptides could be a therapeutic strategy for allergic disease because Th2 cells are responsible for the IgM to IgE switch and subsequent IgE Ab production. Alternatively, other peptides might induce Th1 cells, which may result in the formation of IgG anti-allergen Abs that compete with IgE for allergen and could therefore have a beneficial effect on allergic reactions. However,

both in our study of *Lol p I* and in the birch pollen study (8), the Th1 or Th2 type of lymphokine secretion pattern did not correlate with any T cell peptide epitopes, suggesting that it is unlikely that the Th1:Th2 cell ratios can be affected by immunization or tolerization with selected synthetic peptides.

Briner et al. (4) recently reported that s.c. injections of two synthetic peptides representing the dominant T cell epitopes of the cat allergen *Fel d I* induced inhibition of both the primary and secondary immune response to the antigenic polypeptide chain 1 of the *Fel d I* allergen in mice. These authors concluded that it is not necessary to tolerize all of the T cells reacting with *Fel d I* peptide epitopes to suppress the immune response to this allergen. Whether induction of T cell anergy by s.c. injection of a limited number of peptides from the pollen allergens *Lol p I* and *Bet v I* or the house dust mite allergen *Der p II* will induce suppression of the immune response to these allergens will have to be tested in experimental animals.

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