

Identification of T-cell epitopes of Lol p 9, a major allergen of ryegrass (*Lolium perenne*) pollen

Bella Blaher, PhD,^a Cenk Suphioglu, PhD,^b R. Bruce Knox, DSc,^b
Mohan B. Singh, PhD,^b James McCluskey, MB BS,^c and
Jennifer M. Rolland, PhD^a *Prahran and Parkville, Victoria
and Bedford Park, South Australia, Australia*

T-cell recognition of Lol p 9, a major allergen of ryegrass pollen, was investigated by using a T-cell line and T-cell clones generated from the peripheral blood of an atopic donor. The T-cell line reacted with purified Lol p 9, as well as with crude ryegrass pollen extract, but failed to cross-react with Bermuda grass pollen extract. All of six T-cell clones generated from this line proliferated in response to Lol p 9. Epitope mapping was carried out with a panel of 34 overlapping synthetic peptides, which spanned the entire sequence of the Lol p 9 12R isoform. The T-cell line responded to two of the peptides, Lol p 9 (105-116) and Lol p 9 (193-204), whereas reactivity with one or other of these peptides was shown by five T-cell clones. These two peptides contained sequences consistent with motifs previously reported for major histocompatibility complex class II-restricted peptides. HLA antibody blocking studies showed that presentation of peptide Lol p 9 (105-116) to one T-cell clone was HLA-DR-restricted; this clone expressed a T helper cell phenotype (CD3⁺, CD4⁺) and the T-cell receptor $\alpha\beta$. The identification of immunodominant T-cell epitope(s) on allergens is essential for devising safer and more effective immunotherapy strategies, which can interrupt the chain of events leading to allergic disease. (J Allergy Clin Immunol 1996;98:124-32.)

Key words: Allergy, T cells, ryegrass pollen, Lol p 9, peptides, MHC

The importance of grass pollens as respiratory allergens causing allergic immune responses is well recognized, and several components of pollen grains have been shown to be responsible for these reactions.¹ Ryegrass (*Lolium perenne*) pollen causes symptoms in allergic individuals during the grass flowering season in cool, temperate climates.^{2,3} Lol p 1 and Lol p 9 are recognized by more than 90% of sera of patients allergic to ryegrass pollen.⁴ Originally designated Lol p Ib⁴ because of its similarity in molecular weight to Lol

Abbreviations used

APCs:	Antigen-presenting cells
cpm:	Counts per minute
MHC:	Major histocompatibility complex
PBMCs:	Peripheral blood mononuclear cells
SI:	Stimulation index
TCR:	T-cell receptor

p 1, Lol p 9 is now recognized as a distinct component of ryegrass pollen lacking any sequence homology with Lol p 1.⁴⁻⁷

Current knowledge of grass pollen allergenic determinants is based largely on antibody binding studies.⁸⁻¹⁰ However, it is clear that T cells play a pivotal role in regulating the type and degree of immune reactivity to antigens.¹¹ An early critical event in the allergic immune response is T-cell recognition of allergenic peptides presented in association with major histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells (APCs).^{12, 13} The immune re-

From ^aDepartment of Pathology and Immunology, Monash University, Prahran, Victoria, Australia; ^bSchool of Botany, University of Melbourne, Parkville, Victoria, Australia; and the ^cFlinders Medical Centre, Bedford Park, South Australia, Australia.

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Reprint requests: Jennifer M. Rolland, PhD, Department of Pathology and Immunology, Monash University, Prahran, Victoria 3181, Australia.

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sponse is therefore controlled first by the ability of the APCs to process allergen and present resultant peptides bound to the individual's MHC molecules. Second, in order for an immune response to ensue, T cells with the appropriate receptors that can interact with this peptide-MHC complex must be present.^{14, 15} After binding of the T-cell receptor (TCR) to the peptide-MHC complex, the activated T cell acquires functional properties and produces cytokines that regulate the B-cell response to the allergen.¹⁶ In particular, B-cell proliferation and differentiation into IgE-producing plasma cells is IL-4-dependent.¹⁷

Selective MHC restriction of peptide presentation to T cells (MHC restriction) is one mechanism that might explain HLA phenotype associations with antibody response to allergens in population studies. For example, in the case of ryegrass pollen allergens, IgE antibody responses to Lol p 1 and Lol p 2 have been shown to be associated with HLA-DR3,¹⁸ and IgE responses to Lol p 3, with HLA-DR3 and HLA-DR5.¹⁹ Furthermore, Lol p 3 antibody responsiveness has been shown to be associated with a particular amino acid sequence, EYSTS, found in some DR molecules including DR3.²⁰ However, few studies have investigated MHC restriction of responsiveness to allergens at the T-cell level. In one such study, O'Hehir et al.²¹ found that HLA-DRw52 (an HLA-DRB3 gene product) was the predominant MHC restriction element for the T-cell response to *Dermatophagoides farinae* by an atopic donor.

We have previously studied peripheral blood mononuclear cell (PBMC) responses to ryegrass pollen in atopic and nonatopic subjects,²² and in this study we further examine the T-cell epitope specificity of Lol p 9. A T-cell line and several T-cell clones have been generated from an atopic donor, and T-cell epitopes have been mapped by using overlapping Lol p 9 peptides. The MHC restriction of T-cell recognition of one of these peptides has also been investigated. Knowledge of T-cell epitopes of allergens is of considerable importance for the refinement of immunotherapy programs to modulate the immune responsiveness to these allergens. Current programs use repeated small doses of crude pollen extract, but understanding of their mode of action is limited.^{23, 24} The small but real chance of adverse reactions and the long time needed to reach clinical efficacy, if achieved at all, highlight the need to improve the

risk- to-benefit ratio of allergen-specific immunotherapy.^{25, 26}

METHODS

Donor

The T cell donor for this study (RT: HLA phenotype DR4, DR53, DQ3) was a male Caucasian with seasonal rhinitis and positive skin test (10 mm) and RAST (grade 3) responses to ryegrass pollen. This study was approved by the Alfred Hospital Ethics Review Committee.

Allergen preparations

Ryegrass and Bermuda grass (*Cynodon dactylon*) pollens were obtained from Greer Laboratories, Inc. (Lenoir, N.C.) as dry nondefatted preparations. Crude pollen extracts were prepared with 1 mmol/L NH_4HCO_3 (10% wt/vol). After centrifugation and dialysis, supernatants were then passed through a 0.20 μm filter, and protein concentrations were determined by using the Bio-Rad Microassay (Bio-Rad Laboratories, Life Science Group, Hercules, Calif.).

Lol p 9 was purified from crude ryegrass pollen extracts by fluid-phase liquid chromatography. Briefly, the extract was first subjected to a buffer change from phosphate-buffered saline to 20 mmol/L Tris buffer, pH 12.0, (buffer A) on an Econo-Pac 10DG column (Bio-Rad Laboratories, Life Science Group). A volume of extract containing 5 mg of protein was then injected onto an ion-exchange fluid-phase liquid chromatography column (Mono Q HR 5/5; Pharmacia, Uppsala, Sweden), which had been pre-equilibrated sequentially with buffer A for 5 minutes, buffer B (20 mmol/L Tris, pH 7.0) for 10 minutes, and finally buffer A for an additional 5 minutes. The column was washed with buffer A for 10 minutes to allow for the binding of Lol p 9 and for the elution of other proteins with isoelectric point values equal to or greater than 12. Elution of Lol p 9 was then performed with buffer B at a constant flow rate of 1 ml/min for 25 minutes at room temperature. Fractions of 1 ml were collected every minute and then analyzed by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and immunoblotting with a Lol p 9-specific monoclonal antibody.²⁷ Fractions containing Lol p 9 were pooled, and the protein concentration was determined.

On the basis of the amino acid sequence of the Lol p 9 12R isoform, deduced from the cDNA sequence,^{28, 30} 12-mer and four 13-mer solid-phase peptides with a four- or five-amino acid overlap were synthesized on a BT7400 manual peptide synthesizing block (Biotech Instruments Ltd., Luton, U.K.). The peptides were synthesized on carboxy-terminal Fmoc-protected amino acid resins (Auspep, Parkville, Victoria, Australia). The resins were first washed and drained sequentially three times in 1 ml of dimethyl-formamide (Auspep) and then treated with 1 ml of "deprotecting" solution (25% piperidine in dimethyl-formamide, Auspep) for 10 minutes to remove the

F-moc group from the first carboxy-terminal amino acid. The resins were again washed and drained sequentially three times with 1 ml of dimethylformamide, twice with 1 ml of dichloromethane (Auspep) and finally twice with 1 ml of dimethylformamide. Ten-fold excess of the selected amino acids was combined with activating reagent (Auspep), dissolved in 1 ml of activating solution (Auspep), and added to the appropriate wells for coupling. After a 6-hour coupling procedure at room temperature, the next amino acid was added in the same way. The procedure was repeated until the desired peptide length was achieved. The peptides were then treated with cleaving solution (95% trifluoroacetic acid, 4% phenol, and 1% ethanedithiol; Auspep) for 6 hours to facilitate peptide cleavage from the resin beads. The wells were then washed three times with 1 ml of trifluoroacetic acid, and the collected peptides were then precipitated in 50 ml of cold diethyl ether (Auspep) and isolated on Whatman no. 1 filter papers (Whatman Plc, Maidstone, Kent, U.K.). These filter papers were washed with 50 ml of lyophilizing solution (Auspep) and freeze-dried. Peptides were subjected to analysis by high-performance liquid chromatography and mass spectrometry by Auspep Laboratories (Australia) to check peptide purity and amino acid composition. The peptide Lol p 9 (97-108) was produced in very small amounts only and therefore could not be tested in this study.

Generation of allergen-reactive T-cell line and clones

PBMCs were isolated from heparinized blood samples by centrifugation on a Ficoll-Paque (Pharmacia) density gradient and cultured at 1×10^6 cells/ml with Lol p 9 (2 μ g/ml) in 96-well round-bottom microtiter plates (ICN Flow Laboratories, McLean, Va.). Culture medium comprised RPMI-1640 (ICN Biomedicals, Costa Mesa, Calif.) tissue culture medium supplemented with 1% L-glutamine (ICN), 0.125% gentamicin (Delta West, Bentley, Australia), and 5% heat-inactivated human A+ serum; and cultures were performed at 37°C in 5% CO₂ atmosphere. Seven days after initiation of culture, 10 U/ml human recombinant (r)IL-2 (Chiron Therapeutics, Emeryville, Calif.) was added to the wells. On day 12, the cells were pooled and re-plated at 2.5×10^5 /ml in a 24-well tissue culture plate (Corning Costar Corp., Cambridge, Mass.) in the presence of 2.5×10^5 /ml irradiated (2000 rad) autologous PBMCs, 2 μ g/ml Lol p 9, 20 U/ml rIL-2, 5 to 10 U/ml Lymphocult (Biotest AG, Dreieich, Germany), and 0.1 μ g/ml phytohemagglutinin (Wellcome Plc, London, U.K.). The line was restimulated in this way every 7 to 8 days. On day 20, T cells were cloned by limiting dilution of the line (3000 to 0.3 cells/well). The clones were maintained by addition of 10 to 20 U/ml rIL-2 every 4 days and addition of irradiated autologous PBMCs, 2 μ g/ml Lol p 9, 20 U/ml rIL-2, and 10 U/ml Lymphocult every 7 to 8 days. Before use in proliferation assays, the line and clones were rested

for 7 days after the last addition of irradiated autologous PBMCs, allergen, and IL-2.

Proliferation assays

T cells (5×10^5 /ml) were cultured with 5×10^5 /ml irradiated (2000 rad) autologous PBMCs and Lol p 9 (0.625 to 20 μ g/ml) or Lol p 5 peptides (10 μ g/ml) in 96-well round-bottom microtiter plates for 88 hours, then pulsed with 1 μ Ci/well tritiated thymidine (Amersham Corp., Arlington Heights, Ill.) for 8 hours before harvesting onto glass fiber filters. Control cultures included cells with no allergen, cells with phytohemagglutinin (2 μ g/ml), T cells alone, and irradiated autologous PBMCs alone. The counts per minute (cpm) for each filter were determined by using a liquid scintillation counter (Wallac OY, Turku, Finland). Results are expressed as the mean cpm for replicates or as a stimulation index (SI), the ratio between cpm for cultures with T cells plus irradiated APCs plus antigen and cpm for cultures containing T cells and irradiated APCs alone. An SI greater than or equal to 2 was considered positive.²²

Immunophenotyping

Cells were stained by direct or indirect immunofluorescence with the following monoclonal antibodies: CD2 (Dakopatts A/S, Copenhagen, Denmark); CD3, CD4, CD5, CD8, CD19, TCR $\alpha\beta$, and TCR $\gamma\delta$ (Becton Dickinson Immunocytometry Systems, San Jose, Calif.), CD11b, CD45RO, and CD45RA (Dakopatts). For indirect immunofluorescence, fluorescein isothiocyanate-conjugated sheep anti-mouse Ig F(ab')₂ (Silenus Laboratories Pty. Ltd., Hawthorn, Victoria, Australia) was used, and normal mouse immunoglobulin was included as a negative control. All cells were stained with propidium iodide to permit exclusion of dead cells and were analyzed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems).

MHC restriction studies

To determine the MHC restriction of T-cell clone A12 recognition of Lol p 9 (105-116), the effect of addition of anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP monoclonal antibodies (Becton Dickinson Immunocytometry Systems) on the proliferative response to the peptide was tested. T cells were cultured for 96 hours at 1×10^5 cells/ml with 1×10^5 cells/ml irradiated autologous PBMCs and 10 μ g/ml Lol p 9 (105-116) in the presence or, as a control, absence of 5 μ l/ml anti-HLA antibody. Proliferation was assessed by tritiated thymidine uptake as before.

RESULTS

Allergen specificity of a Lol p 9 T-cell line

We reasoned that the donor (RT) might have T cells reactive to Lol p 9 in view of the clinical history, skin test reactivity, and RAST positivity to ryegrass pollen extract. Thus a bulk helper T-cell line was generated by stimulating PBMCs with Lol p 9 and growth factors.

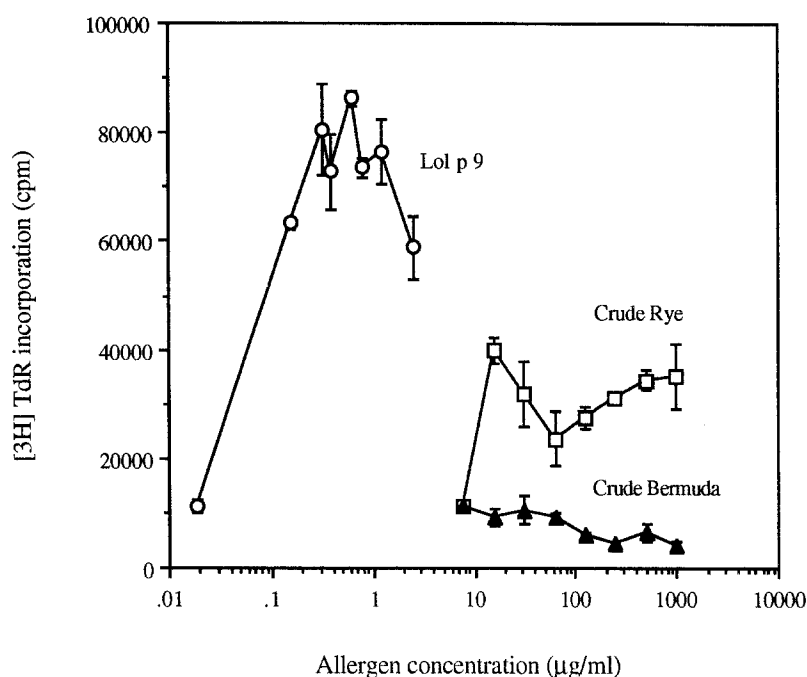


FIG. 1. Allergen specificity of the Lol p 9 T-cell line. Cells were cultured with Lol p 9 and crude ryegrass and Bermuda grass pollen extracts for 4 days. Reactivity was assessed by tritiated thymidine incorporation, and the mean cpm $\pm$ SD for triplicate wells are shown. The maximum SI response to these allergens was 7.8 for Lol p 9, 3.5 for ryegrass pollen extract, and 1.8 for Bermuda grass pollen extract. The background count in the absence of allergen was 11,300 $\pm$ 1246 cpm. [3H]TdR, Tritiated thymidine.

The T-cell line showed strong proliferative reactivity to Lol p 9 and crude ryegrass pollen extract but did not respond to crude Bermuda grass pollen extract (Fig. 1). When this T-cell line was assessed for reactivity to the Lol p 9 peptide series, there was a clear response to Lol p 9 (105-116) and a weaker response to Lol p 9 (193-204) (Fig. 2). To evaluate the clonal specificity of T cells within this line, T-cell clones were isolated by limiting dilution.

T-cell epitope mapping of Lol p 9

Six T-cell clones isolated from the T-cell line were stimulated with the overlapping Lol p 9 peptides. Four patterns of reactivity were observed. First, a strong response to only Lol p 9 (105-116) was exhibited by the T-cell clone A12 (Fig. 3, A). Second, a moderate response to Lol p 9 (193-204) and a weak response to the neighboring peptide (201-212) were demonstrated by two clones, C2 and C3 (Fig. 3, B). Third, weak reactivity to only Lol p 9 (105-116) was shown by two clones (C4 and H3; data not shown). Finally, a lack of response to any peptide, despite a strong response to the whole Lol p 9 allergen, was shown by T-cell clone A3 (Fig. 3, C). These results indicate that one T-cell epitope of Lol p 9 is encompassed

within the Lol p 9 (105-116) region and another is within the region encompassed by Lol p 9 (193-204) and Lol p 9 (201-212).

Immunophenotyping of the T-cell line and clone A12

Immunophenotyping confirmed that both the T-cell line and T-cell clone A12 comprised mature T cells. The majority of the T-cell line cells were CD4⁺ (T helper phenotype) with only a small proportion of CD8 cells, whereas the cloned T cells were exclusively CD4⁺. Interestingly, a small subset of the T-cell line expressed the TCR $\gamma\delta$, but the T-cell clone A12 was uniformly TCR $\alpha\beta$ ⁺. As assessed with the CD45RA and CD45RO markers, naive T cells were not present in these cultures.²⁹

MHC restriction of Lol p 9 (105-116) presentation to T-cell clone A12

To identify the MHC-restricting element presenting Lol p 9 (105-116) to the T-cell clone A12, the effect of addition of anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP monoclonal antibodies on the T-cell response to the peptide was examined. All three of these monoclonal anti-HLA antibodies reacted with a human lymph node cell suspen-

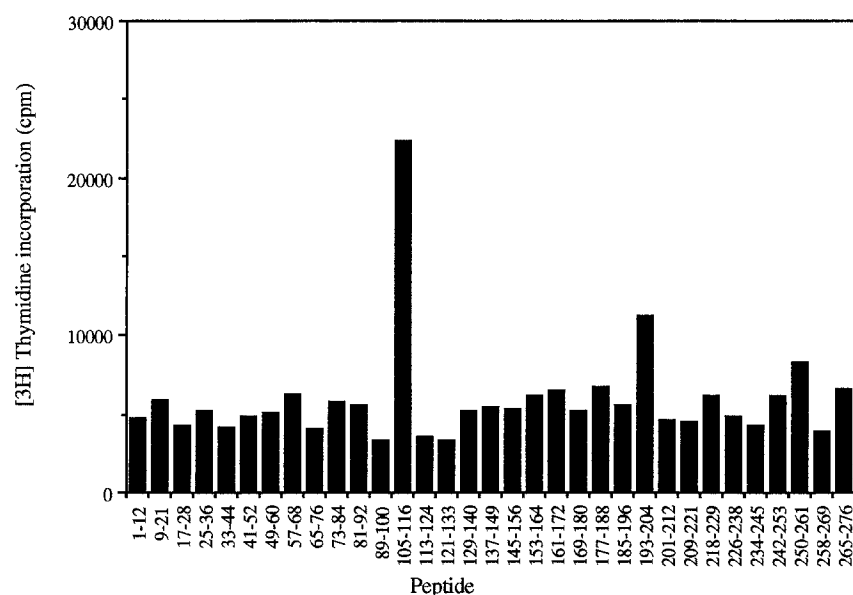


FIG. 2. Proliferative response of the Lol p 9 T-cell line to Lol p 9 synthetic peptides. The peptides were tested at 10 μ g/ml, and results are expressed as the mean cpm of duplicate cultures. The line responded to Lol p 9 (105-116) (SI = 4.5) and weakly to Lol p 9 (193-204) (SI = 2.3). The mean background count for cells cultured without peptide was 4849 cpm.

sion in an immunofluorescence assay (data not shown). Fig. 4 shows the effect of these monoclonal antibodies on the proliferative response of T-cell clone A12 to Lol p 9 (105-116) presented by autologous irradiated PBMCs. In the absence of Lol p 9 (105-116), these antibodies were neither inhibitory nor mitogenic to T cells and APCs. The T-cell response to Lol p 9 (105-116) was completely abolished by anti-HLA-DR but was only marginally inhibited by anti-HLA-DP and was unaffected by anti-HLA-DQ. These results strongly suggest that HLA-DR molecules were the restriction element for recognition of Lol p 9 (105-116) by the T-cell clone A12.

DISCUSSION

We have identified two T-cell determinants on Lol p 9, a major allergen of ryegrass pollen: Lol p 9 (105-116) and Lol p 9 (193-204). These peptide determinants were identified by cloning T cells from a Lol p 9-specific T-cell line and characterizing their reactivity on a panel of synthetic Lol p 9 peptides. The two peptides were both recognized by T cells within the polyclonal T-cell line. The weak reactivity of clones C2 and C3 to the Lol p 9 (201-212) peptide and the strong reactivity to the adjacent peptide Lol p 9 (193-204) suggest that: (1) the core epitope recognized by these clones involves the residues of Lol p 9 (201-204); (2) a different epitope is associated with Lol p 9 (201-

212); or (3) MHC class II molecules bind the same core peptide, but TCR recognition may be influenced by peptide length.

That T-cell clone A3 showed no reactivity when challenged with the Lol p 9 peptide series, despite strong reactivity with the natural protein, suggests that the crucial amino acid sequences were not encompassed by the 12- and 13-mer peptides synthesized for this study. However, the possibility that this clone recognized an epitope related to a different isoform of Lol p 9 cannot be excluded. Our Lol p 9 peptides were synthesized according to the amino acid sequence for the Lol p 9 12R isoform,²⁸ but there are differences in primary sequence between the 12R and 19R Lol p 9 isoforms²⁸; and at least eight different isoforms of Lol p 9 can be identified by two-dimensional gel electrophoresis.¹⁰ Discrepancies between T-cell responses to allergenic isoforms are known to occur with the birch pollen allergen Bet v 1.^{30, 31}

Both peptides Lol p 9 (105-116) and Lol p 9 (193-204) contain tyrosine (Y) at position 3 and threonine (T) or serine (S) at position 8 (Table I). Thus these peptide sequences are consistent with the motifs described for I-E^b binding peptides in the mouse³² and for HLA-DR4 binding peptides in human studies.³³ In addition, they are consistent with features of the HLA-DR1 binding peptide structure identified from crystallization studies³⁴ in which an amino acid with a large hydrophobic side

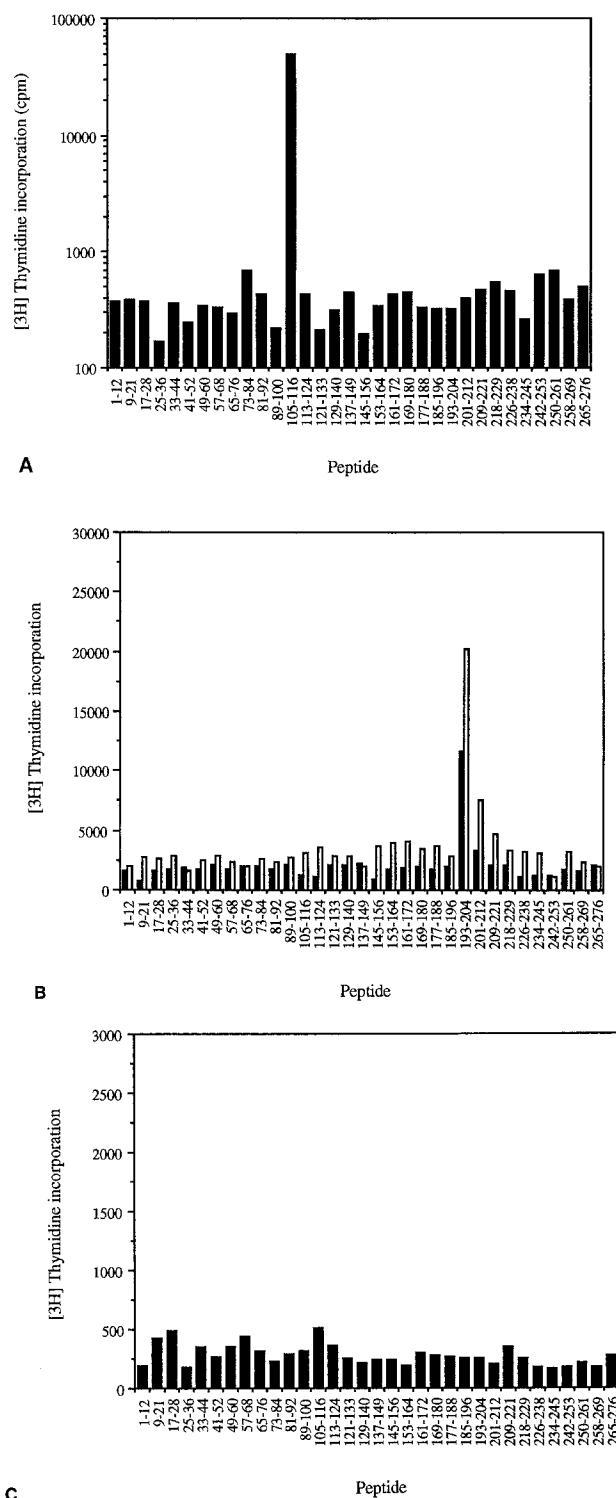


FIG. 3. Proliferative responses of T-cell clones to the Lol p 9 peptide series (10 μ g/ml). Mean cpm of duplicate cultures are shown. **A**, T-cell clone A12 showed strong reactivity to only Lol p 9 (105-116) (SI = 137). Mean background count was 358 cpm. Note: the y axis is shown on a log scale. **B**, T-cell clones C2 (filled bars) and C3 (open bars) responded to Lol p 9 (193-204) (SI = 11 and 8.0, respectively) and weakly to the neighboring peptide Lol p 9 (201-212) (SI = 3.2 and 3.0, respectively). Mean background count for T-cell clone C2 was 1063 cpm, and for T-cell clone C3, 2519 cpm. **C**, T-cell clone A3 showed no response to any of the Lol p 9 peptides. Mean background count was 252 cpm.

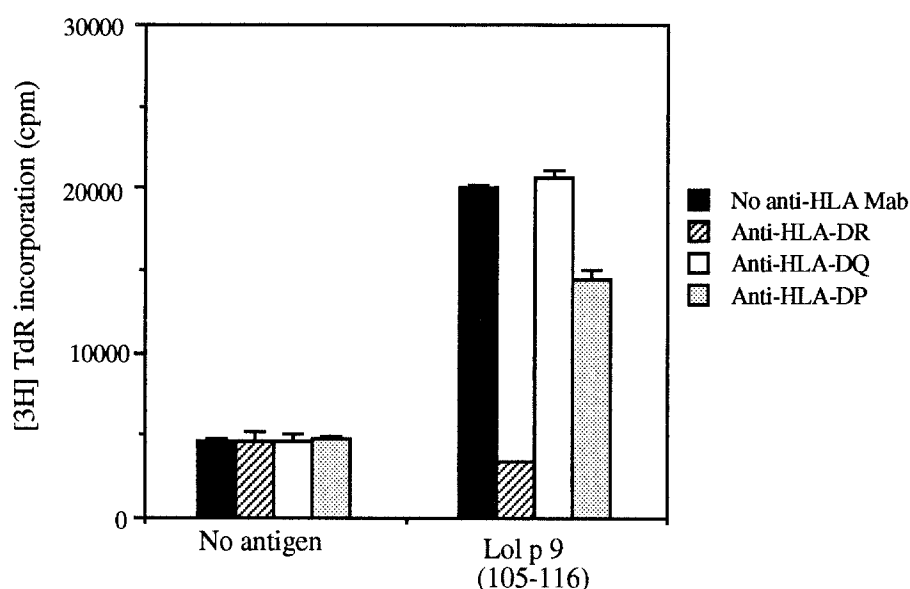


FIG. 4. Effect of anti-HLA monoclonal antibodies on the response to Lol p 9 (105-116) by T-cell clone A12 (145 days old). T cells were cultured at 1×10^5 cells/ml with 1×10^5 cells/ml irradiated autologous PBMCs and $10 \mu\text{g/ml}$ Lol p 9 (105-116) in the presence or absence of $5 \mu\text{l/ml}$ anti-HLA antibody. Proliferation was assessed by the incorporation of tritiated thymidine ($[3\text{H}]\text{TdR}$), and results are expressed as mean cpm for triplicate cultures + SD. *Mab*, Monoclonal antibody.

TABLE I. Amino acid sequences of Lol p 9 dodecapeptides 105-116 and 193-204

Peptide	1	2	3	4	5	6	7	8	9	10	11	12
105-116	A	K	Y	D	A	F	V	T	A	L	T	E
193-204	E	T	Y	K	F	I	P	S	L	E	A	A

chain (tyrosine, phenylalanine, leucine, isoleucine, or tryptophan) appears to fit into a major pocket, and less hydrophobic amino acids fit into minor pockets, thus contributing to the anchoring of the peptide within the class II binding site.^{34, 35} In this study we demonstrated HLA-DR restriction of T-cell recognition of one of the peptides (Lol p 9 [105-116]). HLA-DR typing of the T cell donor revealed probable DRB1*0401/0408 heterozygosity; thus, three DR alleles could potentially present the Lol p 9 (105-116) peptide in this patient. DR53 presentation seems unlikely because of the lack of presentation of this peptide to clone A12 by another HLA homozygous B-lymphoblastoid cell line expressing DR53 but not DR4 (data not shown). Thus the Lol p 9 (105-116) epitope was most likely DR4-restricted.

The lack of detectable T-cell cross-reactivity between the Lol p 9 and Bermuda grass pollen extracts in this study is consistent with the lack of serologic cross-reactivity between major ryegrass and Bermuda grass pollen allergens documented

in the literature.^{10, 36} However, the observed close correlation between clinical sensitivity to these two grasses suggests that a more extensive investigation of possible T-cell cross-reactivity is warranted.

The specificity of T cells reactive with common allergens is a major area of research effort in allergy at present. However, knowledge of the events that result in preferential activation of allergen-specific T cells that produce IL-4 (i.e., T_{H2} -like T cells) rather than interferon- γ (i.e., T_{H1} -like T cells) is also crucial for understanding the different responses elicited by atopic and non-atopic individuals. In studies by O'Hehir et al.³⁷ house dust mite-reactive T-cell clones isolated from the PBMCs of nonatopic individuals failed to support IgE synthesis, whereas the T cells from atopic donors were able to do so. In studies on the T-cell line described here, we initially found IL-2 in the culture supernatant, but with longer culture time, levels of this cytokine decreased and IL-4 levels increased, consistent with either a change from a T_{H0} to a T_{H2} phenotype with continued

antigenic stimulation or overgrowth of T_{H2} cells in the culture with time.³⁸

This study has identified two Lol p 9 peptides recognized by T cells from a ryegrass pollen-sensitive donor. MHC restriction of the T-cell response to one of these peptides was demonstrated. Whether these T-cell epitopes were identified as a result of a cloning bias and whether they are also recognized by T cells from other atopic donors should now be examined by testing peripheral blood reactivity of a large series of donors. A comparison of T-cell epitopes recognized by atopic and nonatopic donors, both in and out of the pollen season, would reveal clinically relevant peptides. In addition, further characterization of the responding T cells in terms of their expression of CD4 or CD8 and their MHC restriction will lay the foundations for devising vaccines to immunomodulate the allergic immune response to ryegrass pollen.

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