

T CELL ACTIVATION-INDUCING EPITOPES OF THE HOUSE DUST MITE ALLERGEN *Der p* I

Proliferation and Lymphokine Production Patterns by *Der p* I-Specific CD4⁺ T Cell Clones

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Cloned human CD4⁺ T cell lines specific for the house dust mite *Dermatophagoides pteronyssinus* were used to map minimal T cell activation-inducing epitopes on the Group I allergen in *D. pteronyssinus* extracts (*Der p* I) molecule. Most of these *Der p* I-specific T cell clones expressed different TCR α and β gene products. Using recombinant deletion proteins, three T cell epitopes were identified on the *Der p* I molecule; p45-67 and p117-143 were recognized by HLA-DR7-restricted T cells, whereas p94-104 was recognized in the context of HLA-DR2, -DRw11 (DR5), and -DR8 molecules. This degenerate class II MHC restriction appears to be due to shared Phe and Asp residues at positions 67 and 70, respectively, in the third variable domain of the HLA-DR β chain. All three T cell epitopes induced Th2-like cytokine production profiles by the *Der p* I-specific T cell clones, which were characterized by the production of very high levels of IL-4 and IL-5, as compared with those secreted by tetanus toxin-specific T cell clones derived from the same patients, but no or low amounts of IL-2 and IFN- γ . This Th2-like production profile was, however, not an intrinsic property of the *Der p* I-specific T cells, but was dependent upon their mode of activation. Stimulation with Con A also induced very low or no measurable levels of IL-2 and IFN- γ , whereas activation with TPA and the calcium ionophore A23187 resulted in the production of high levels of IL-4, IL-5, IL-2, and IFN- γ . These results indicate that *Der p* I-specific T cell clones are not defective in their capacity to produce high levels of Th1 cytokines.

Two closely related mite species of the genus *Dermatophagoides*, *pteronyssinus* and *Dermatophagoides farinae*, have been shown to be responsible for long-lasting allergic symptoms and diseases such as asthma.

atopic dermatitis, and perennial rhinitis (1, 2). Many studies have dealt with the characterization of the allergens they produce. Two proteins, excreted by *Der pt*³ and *Der f* (3) and designated *Der p* I and *Der f* I, respectively, were found to represent a homologous pair of major allergens, both with a m.w. of 24 kDa (4, 5). Both allergens have been purified and 70% to 80% of patients allergic for house dust mite were found to have IgE antibodies directed against *Der p* I (6, 7). IgE and IgG antibodies from humans or mice were found to detect cross-reacting, as well as species-specific epitopes (5). The cDNA coding for the *Der p* I allergen has been cloned and expressed (8). Sequence analysis has revealed that *Der p* I has a high degree of homology both at the nucleotide and the amino acid level with actinidin and papain, which belong to the group of cysteine proteases (9).

The *Der p* I allergen induces proliferative responses by peripheral blood T cells from the majority of patients who are allergic to house dust, but not in control nonatopic donors (10). In addition, cloned T cell lines, reactive with *Der pt* (11) and *Der f* (12, 13) that were all restricted by HLA-DR-encoded molecules have been described. In addition to the major HLA-DRB1 gene products, the super-typic HLA molecule HLA-DRw52, which is a HLA-DRB3 gene product, has also been shown to play a functional role in the recognition of house dust mite allergens by Th lymphocytes (14). In addition, it has been reported that a minority of the *Der f*-specific T cell clones cross-reacted with determinants present on *Der pt* (13).

The analysis of immunodominant T cell antigenic sites on the *Der p* I molecule is important for our understanding of the T cell responses in *Der p* I-induced allergic reactions. Furthermore, protein sequences that elicit T cell Ir have potential applications in future therapies, such as vaccine development, or may have diagnostic value when used in skin testing of atopic patients. However, in spite of extensive serologic studies on the epitope specificity of human IgE antibodies, reactive with *Der p* I and *Der f* I (4, 5, 15), no studies to date have dealt with the analysis of epitopes on the *Der p* I molecule that can induce lymphokine production and proliferative responses by *Der p* I-reactive T cells.

³ Abbreviations used in this paper: *Der pt*, *Dermatophagoides pteronyssinus* (house dust mite); *Der f*, *Dermatophagoides farinae*; *Der p* I, group I allergen in *Der pt* extracts; EBV-LCL, Epstein-Barr virus transformed B cell line; TT, tetanus toxin; TPA, 12-O-tetradecanoylphorbol 13-acetate; PCR, polymerase chain reaction.

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Therefore, we determined the T cell activation-inducing epitopes on the *Der p 1* antigen, using cloned *Der p 1*-specific human T cell lines obtained from two atopic patients (HLA-DRw11 and HLA-DR4,7, respectively) allergic to *Der pt*. In addition, we have characterized the functional properties of these *Der p 1*-reactive T cell clones and show that they have a Th2-like cytokine production phenotype after activation by *Der p 1* or Con A, but that they are not defective in their capacity to produce the Th1 cytokines IL-2 and IFN- γ .

MATERIALS AND METHODS

Patients. N.P. (HLA DRw11,-; DRw52; DQw3) and A.C. (HLA DR4,7; DRw53; DQw3) are atopic patients who suffer from allergic asthma and severe atopic dermatitis and who are clinically allergic to *Der pt*. Radioallergosorbent tests (Class 4: >17.5 U) indicated high titers of *Der pt*-specific IgE antibodies in the serum of both patients.

T cell clones and cell lines. Cloned *Der p 1*-specific T cell lines were generated from patients N.P. and A.C. by stimulating their PBMC with 1 μ g/ml of a lyophilized extract of *Der pt* (Diephuis Laboratories, Groningen, The Netherlands). Ten days later, growing cells were cloned by limiting dilution at concentrations of 0.3 cells/well in 96-well round-bottomed Linbro plates (Flow Labs, McLean, VA), in the presence of 5×10^5 /ml irradiated (4000 rad) allogeneic PBMC, 5×10^4 /ml irradiated (5000 rad) autologous EBV-LCL, and 0.1 μ g/ml purified PHA (Wellcome Diagnostics, Dartford, United Kingdom). After 14 days, growing cultures were transferred to 24-well Linbro plates and were restimulated every 14 days with a feeder mixture consisting of irradiated allogeneic PBMC, irradiated allogeneic EBV-LCL, and PHA, as described previously (16). Three to 4 days after each restimulation, the cultures were split and further expanded in medium containing 20 U/ml rIL-2. Control T cell clones were from the same donors and had been generated by stimulation of PBMC with 1 μ g/ml TT (a generous gift of Dr. Bizzini, Institut Pasteur, Paris, France) and cloning by limiting dilution, following the protocol described above.

Autologous and allogeneic HLA-typed EBV-LCL, which were used as APC, were prepared from PBMC by in vitro infection with EBV obtained from the marmoset cell line B95-8, in the presence of 1 μ g/ml cyclosporin A (Sandoz, Basel, Switzerland). HLA-typed EBV-LCL were generously provided by Dr. Jonathan Rothbard (Immunologic, Palo Alto, CA). Amino acid sequence data of the β -chains of HLA-DR2 (17, 18), DR-w11 (18, 19), and DR8 (18) have been published. Murine fibroblasts, transfected with genes coding for HLA-DR7, HLA-DRw52a, HLA-DRw52b, and HLA-DRw52c (a generous gift of Dr. Inoko, Tokai University, Kanagawa, Japan) were used as an additional source of HLA-DR-typed APC. Transfected fibroblasts were cultured in RPMI 1640 medium supplemented with 0.01 mM β -ME, 10% FCS, and 500 μ g/ml of antibiotic G418 for continued selection. All cloned T cell lines and EBV-LCL were cultured in a modification of Iscove's medium, described by Yssel et al. (20), supplemented with 1% human AB⁺ serum.

Flow cytometry. Analysis of cells for expression of cell surface Ag was performed by flow cytometry, using a FACScan (Becton Dickinson, Mountain View, CA), as previously described (21). T cell clones were analyzed for the expression of T cell surface Ag using anti-CD2 (Leu 5), anti-CD3 (Leu 4a), anti-CD7 (Leu 9), anti-CD8 (Leu 2a), and anti-CD45 mAb, all purchased from Becton Dickinson.

Proliferation assays. Cellular proliferation was measured using a [³H]TdR assay, as has been previously described (21). In brief, 2×10^4 T cells that had been rested for 10 to 12 days after the last restimulation with feeder cells were mixed with different amounts of irradiated APC, in the presence or absence of Ag, in round-bottomed 96-well plates (Linbro) and in a total volume of 200 μ l. After 72 h of incubation at 37°C, 1 μ Ci [³H]TdR (Amersham, Arlington Heights, IL) was added to each well. Four h later, the cells were harvested onto glass fiber strips, and the amount of incorporated [³H]TdR was measured by liquid scintillation counting. The results are expressed as the mean of triplicate cultures $\pm$ the SD. The inhibitory effects of anti-HLA class II mAb were determined by preincubation of the APC with various dilutions of the anti-HLA DR/DP mAb Q5/13 (22) or the anti-HLA-DQ mAb SPV-L3 (23) for 1 h before the addition of the T cells.

Stimulation of T cell clones and assays for cytokine production. Cytokine-containing T cell supernatants were prepared from T cells 10 to 14 days after their last restimulation with feeder cells. One million T cell clones that had been washed twice with medium were stimulated with various amounts of recombinant *Der p 1* or synthetic *Der p 1* peptides in the presence of 10^6 irradiated APC in 24-well

plates (Linbro), in a final volume of 1 ml. After 18 h of incubation, supernatants were harvested, spun at 250 $\times$ g, aliquoted, and stored at -20°C before testing. Levels of IL-4 (24), IL-5 (25), and IFN- γ (26) were determined by ELISA. IL-2 was also measured in an ELISA in which the anti-IL-2 mAb BG-5 (kindly provided by Dr. J. Wijdenes, Centre de Transfusion Sanguine, Besançon, France) was used as a coating antibody, and a polyclonal goat anti-IL-2 antiserum was used as a catcher antibody. This ELISA is specific for IL-2 and does not detect any of the known recombinant human cytokines. The sensitivity of the assay was 10 pg/ml (H. Yssel, unpublished observations).

Determination of TCR $V\alpha$ and $V\beta$ gene products. Total RNA was prepared from 20×10^6 cells by guanidium thiocyanate lysis followed by CsCl centrifugation as described (27). Two micrograms of total RNA were reverse transcribed, using 400 U M-MuLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and random hexa nucleotides (250 pmol) for 1 h at 37°C in a 100 μ l reaction. Each cDNA sample was subjected to two rounds of amplification using 5 μ l of cDNA per reaction. In the first round, the cDNA were amplified in four independent reactions using a C β -specific primer and pools of $V\beta$ -specific primers as follows: pool 1 contained $V\beta 1$, $V\beta 2$, $V\beta 3$, $V\beta 4$, 5:1; pool 2 contained $V\beta 5.2-3$, $V\beta 6.1-3$, $V\beta 7$, $V\beta 8$, $V\beta 9$, $V\beta 10$; pool 3 contained $V\beta 11$, $V\beta 12$, $V\beta 13.2$, $V\beta 14$, $V\beta 15$; and pool 4 contained $V\beta 16$, $V\beta 17$, $V\beta 18$, $V\beta 19$, $V\beta 20$. After detection of an amplified product, the cDNA were amplified in the second round, using the individual $V\beta$ primers from the corresponding pool. PCR conditions were as suggested by the manufacturer (IBI, New Haven, CT). The amplification reactions were performed with 0.5 U Taq polymerase (Cetus Corporation, Emeryville, CA) and 0.5 mM primers in 100- μ l reactions, using a Cetus/Perkin-Elmer thermocycler under the following conditions, as suggested by the manufacturer: 95°C melting for 1 min, 55°C annealing for 2 min, and 72°C extension for 3 min. The sequences of the $V\beta$ and C β primers were as described by Choi et al. (28).

Synthesis of *Der p 1* peptides. Synthetic peptides, based on the amino acid sequence of the *Der pt* cDNA, were synthesized using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA), using a standard t-BOC chemistry program. The peptides were passed over a G10-Sephadex column (Pharmacia, Uppsala, Sweden) and the peptide fraction was further purified as a single peak by HPLC on a preparative C8 column with a solvent system of 0.1% TFA and an increasing gradient of acetonitrile. The purity of the cleaved peptides was checked with reverse-phase HPLC and the N-terminal amino acid sequence was determined. None of the synthesized peptides was found to be toxic for APC, even at concentrations up to 10 μ g/ml.

Construction, expression, and purification of recombinant *Der p 1* and *Der p 1* deletion mutants. *Der p 1* DNA (8) (a kind gift of Dr. W. Thomas, Princess Margaret Hospital for children, Perth, Australia) was expressed in *Escherichia coli*, using the fusion vector pGEX-2T (29). *E. coli* AB1899 cells carrying pGEX2T-*Der p 1* or *Der p 1* mutant plasmids were grown in L broth (1:50 dilution of an overnight culture) for 2 h at 37°C. Isopropyl- β -D-galactopyranoside was added to a final concentration of 0.1 mM and, after 3 to 4 h of subsequent culture, the cells were harvested by centrifugation. Cells were lysed by sonication in PBS buffer in the presence of 0.5% Triton X-100 and 0.1 mM PMSF. After centrifugation at 8000 $\times$ g for 30 min, the supernatant was mixed with glutathione Sepharose 4B beads (Pharmacia). After absorption for 2 min at room temperature, beads were collected by centrifugation for 2 min at 500 $\times$ g and washed five times with PBS supplemented with 0.5% Triton. Recombinant *Der p 1* protein and *Der p 1* mutant proteins were recovered by incubation of the glutathione Sepharose 4B bead solution with 50 U of thrombin in 100 mM Tris-HCl pH 8, 150 mM NaCl, and 2.5 mM CaCl₂ for 1 h at 37°C, resulting in the release of *Der p 1* in the supernatant. Alternatively, small *Der p 1* mutant proteins (<50 amino acids) were eluted from the beads directly as fusion proteins in 5 mM glutathione without thrombin cleavage. *Der p 1* mutants were generated by PCR techniques. PCR primers were designed so that the DNA encoding *Der p 1* mutant proteins could be cloned as Bam-EcoRI fragments in the fusion vector pGEX-2T. PCR conditions were as described above. In total, 40 cycles of amplification were carried out. The PCR products were purified using a PCR purification column (Qiagen, Studio City, CA). The purity of *Der p 1* proteins prepared using the above described method is routinely >95%, as estimated by PAGE.

It was found that small deletion proteins could not be recovered after their cleavage from the glutathione fusion protein, probably due to their sensitivity to proteolysis. Since no differences in proliferative responses were measured when *Der p 1*, expressed as a whole fusion protein instead of thrombin-cleaved protein was used (results not shown), all deletion mutants were expressed as fusion proteins, containing glutathione transferase.

RESULTS

Generation of *Der p 1*-specific T cell clones. PBMC of two donors allergic to *Der p 1*, were stimulated with a semipurified preparation of *Der pt* Ag, which contained predominantly *Der p 1* as revealed by immunoelectrophoresis (data not shown). Proliferating T cells subsequently were cloned by limiting dilution. T cell clones that proliferated strongly in response to *Der pt* Ag in the presence of autologous EBV-LCL or autologous PBMC used as APC were selected for further studies. Although CD4⁺, as well as CD8⁺ cells were derived from the PBMC of both patients, only CD4⁺ T cell clones were found to proliferate in response to *Der pt*. All *Der pt*-specific CD4⁺ T cell clones used in this study were CD2⁺, CD3⁺, CD7⁺, CD8⁺, and CD45⁺. Furthermore, Southern blot analysis of each of the T cell clones demonstrated their monoclonal origin (results not shown).

As is shown in Table I, three CD4⁺ T cell clones obtained from patient N.P. (NP-12, NP-14, NP-44) and three CD4⁺ T cell clones of patient A.C. (AC-07, AC-15, AC-39) proliferated in response to *Der pt* Ag, presented by autologous PBMC or autologous EBV-LCL. In addition, these clones were found to proliferate specifically in response to stimulation with *E. coli* expressed r*Der p 1*, in the presence of autologous EBV-LCL (Table I). No proliferative responses were observed when the cells were incubated with Ag or APC alone, whereas the responses were comparable in magnitude when EBV-LCL or autologous PBMC were used as APC. The T cell clones failed to proliferate in response to control Ag (TT, PPD) (data not shown).

T cell epitopes on *Der p 1*. To determine the minimal epitopes on the *Der p 1* molecule that could induce T cell proliferation, a series of deletion mutants that covered the entire *Der p 1* protein were generated. The three *Der p 1*-specific NP T cell clones proliferated in response to stimulation with mutant proteins 1–106 (Fig. 1A), whereas the AC T cell clones recognized at least two sequences on *Der p 1*. The minimal T cell proliferation-inducing sequence on the *Der p 1* molecule recognized by the NP T cell clones was mapped, using small mutant proteins with deletions at the C-terminal as well as the N-terminal sites. As shown in Figure 2A, the deletion mutant that lacked the residue at position 103, but not 104 or 105, was no longer able to induce proliferative responses in NP-12, NP-14, and NP-44. Similarly, at the N terminus, removal of the residue at position 95, but not of position 94, resulted in the abrogation of the response (Fig. 2B). Taken together, these data indicate that the NP

T cell clones react with a minimal activation-inducing sequence on the *Der p 1* molecule, located between residues Tyr⁹⁴ and Cys¹⁰⁴, whereas no other epitope on *Der p 1* is recognized. The AC T cell clones recognized at least two sequences on *Der p 1*. T cell clones AC-07 and AC-15 recognized an epitope located between positions 1–67, whereas p1–45 was negative (Fig. 1B), suggesting that the T cell activation indicating epitopes was located between positions 45–67. This was indeed the case. Deletion mutant protein p45–67 induced proliferation of AC-07 and AC-15 (Table II). On the other hand, T cell clone AC-39 recognized an epitope located between positions 117–143 (Fig. 1B). The primary structure of these sequences, based on the nucleotide sequence published by Chua et al. (9), is depicted in Figure 3. The correct sequence of residues 134–139 is depicted here and differs from the one that was originally published (W. Thomas, personal communication).

TCR $V\alpha$ and $V\beta$ gene usage. The expression of the TCR $V\alpha$ and $V\beta$ gene products by the *Der p 1*-specific T cell clones used in this study is shown in Table III. In spite of the fact that the three NP and three AC T cell clones each recognized specific epitopes on the *Der p 1* molecule, none of them was found to share any TCR $V\alpha$ and $V\beta$ gene products.

Degenerate restriction of HLA-DRw11⁺ *Der p 1*-specific T cell clones. The HLA class II molecules that served as restriction elements for the *Der p 1*-specific T cell clones were determined by blocking studies, using anti-HLA class II antibodies and were further analyzed in proliferation studies using homozygous HLA-typed EBV-LCL or murine fibroblasts transfected with HLA-DR molecules. Blocking studies carried out with mAb, directed at framework Ag of HLA-DR and HLA-DQ, respectively, indicated that the AC, as well as the NP T cell clones, recognized *Der p 1* in the context of an HLA-DR molecule (data not shown). Using a small panel of homozygous HLA-DR and -DQ typed EBV-LCL as APC, it was found that the recognition of *Der p 1* by T cell clones AC-07, AC-15, and AC-39 was restricted by an HLA-DR7 molecule (data not shown). Interestingly, NP-12, NP-14, and NP-44 (Fig. 4) were found to react with the p94–104 sequence, presented by HLA-DR2⁺, HLA-DRw11⁺, and HLA-DR8⁺ APC, with the exception of the HLA-DRw11⁺ EBV-LCL JVM and the HLA-DRw2⁺ cell lines REM and AZH. To rule out the possibility that the HLA-DR β 3 chain gene product DRw52 was contributing to the presentation of *Der p 1* Ag by the HLA-DR2⁺, HLA-DRw11⁺, and HLA-DR8⁺ EBV-LCL, murine fibroblasts expressing HLA-DRw52, -DRw52b, and -DRw52c molecules were

TABLE I
Proliferative responses of cloned human *Der p 1*-specific T cell lines to *Der pt* and r*Der p 1* presented by autologous EBV-LCL or PBMC

Ag ^a	Autologous APC ^b	T Cell Clones					
		NP-12	NP-14	NP-44	AC-07	AC-15	AC-39
		[³ H]TdR incorporation (cpm × 10 ⁻³ ± SD)					
<i>Der pt</i>	EBV-LCL	0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
	PBMNC	11.5 ± 0.9	10.3 ± 0.0	10.3 ± 0.0	36.3 ± 4.5	50.5 ± 2.9	75.3 ± 7.0
<i>Der p 1</i>		13.4 ± 1.2	12.1 ± 0.9	12.5 ± 0.5	41.1 ± 2.9	52.6 ± 2.4	85.1 ± 9.9
		0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
	EBV-LCL	12.6 ± 1.4	11.4 ± 0.7	14.8 ± 2.3	21.4 ± 3.7	34.5 ± 5.2	61.4 ± 5.7
	PBMNC	15.5 ± 1.9	12.3 ± 1.1	15.5 ± 1.8	22.3 ± 1.1	35.5 ± 3.9	62.3 ± 4.5

^a Concentration of *Der pt*: 0.5 µg/ml, dilution *Der p 1*: 1/5000.

^b APC/T cell ratio EBV-LCL and PBMNC: 3:1 and 1:1, respectively.

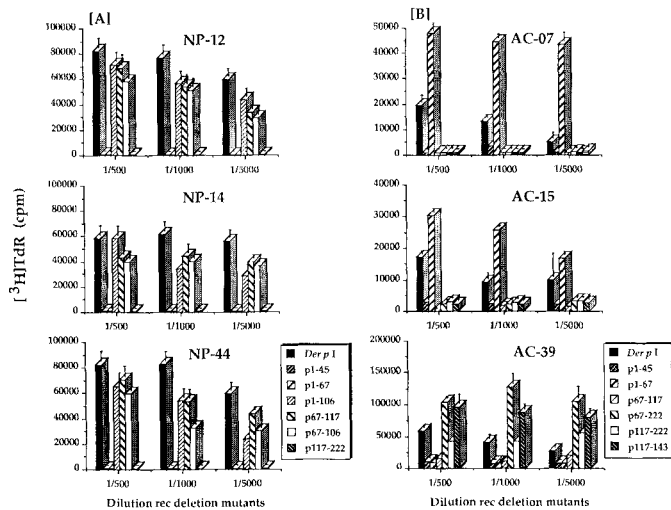


Figure 1. T cell activation-inducing epitopes on *Der p 1* recognized by *Der p 1*-specific T cell clones derived from two atopic patients. *Der p 1*-specific T cell clones NP-12, NP-14, and NP-44 derived from patient N.P. (A) and AC-07, AC-15, and AC-39 derived from patient A.C. (B) were incubated with various dilutions of different deletion mutants, in the presence of autologous EBV-LCL (APC/T cell ratio 3:1) and [^{3} H]TdR was measured after 72 h. Results are expressed as mean $\pm$ SD of triplicate cultures.

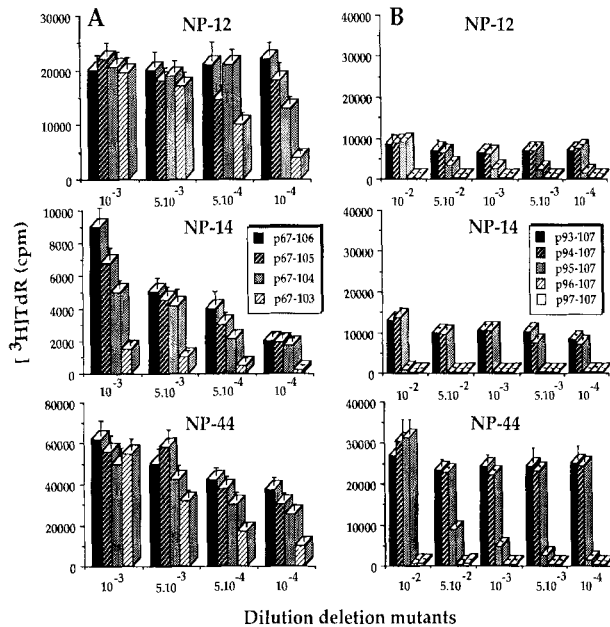


Figure 2. Minimal T cell activation-inducing epitope on *Der p 1* recognized by *Der p 1*-specific T cell clones derived from patient N.P. *Der p 1*-specific T cell clones NP-12, NP-14, and NP-44 derived from patient N.P. were incubated with autologous EBV-LCL (APC/T cell ratio 3:1) and various dilutions of deletion mutants that were deleted at the C- (A) or the N-terminus (B).

TABLE II

Proliferative responses of T cell clones AC-07 and AC-15 to *Der p 1* deletion mutant p45-67

Stimulation	T Cell Clones	
	AC-07	AC-15
	[^{3} H]TdR $\times 10^{-3} \pm$ SD	
Control	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0
<i>rDer p 1</i>	51.3 $\pm$ 4.8	27.5 $\pm$ 3.5
p45-67	45.7 $\pm$ 3.7	38.9 $\pm$ 2.2

used as APC. While control fibroblasts, transfected with DNA coding for HLA-DRw11, were able to present the

45 SAYLAHRNQSLDLAEQELVDCAS 67
 94 YAYVAREQSCR 104
 117 QIYPPNANKIREALQTHSAIAHYWT 143

Figure 3. Amino acid sequence (single letter code) of the T cell activation-inducing epitopes on *Der p 1*.

TABLE III

T cell $V\alpha$ and $V\beta$ gene expression in *Der p 1*-specific T cell clones

T Cell Clone	$V\alpha$	$V\beta$
NP-12	5	13A
NP-14	NT ^a	6
NP-44	6	8
AC-07	3	17
AC-15	3	— ^b
AC-39	NT	—

^a Not tested.

^b Could not be determined from the pool of primers.

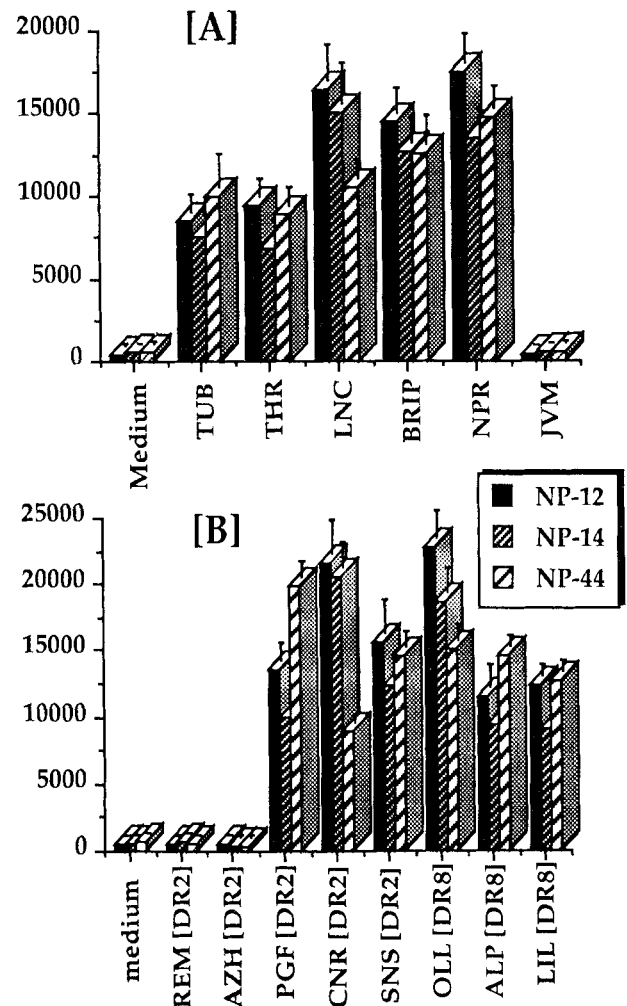


Figure 4. Degenerate restriction of the recognition of *Der p 1* by *Der p 1*-specific T cell clones. *Der p 1*-specific T cell clones NP-12, NP-14, and NP-44 were incubated with 1:1000 dilution of the deletion mutant p94-104, in the presence of HLA-DRw11⁺ (A), or HLA-DR2⁺ and DR8⁺ (B) EBV-LCL (APC/T cell ratio 3:1). [^{3} H]TdR was measured after 72 h.

p94-104 peptide to the NP T cell clones, none of the HLA-DRw52 transfected fibroblasts was able to function as APC (data not shown), indicating that the recognition of *Der p 1* by the NP T cells was restricted only by HLA-DR

β 1 chain products. Comparison of the primary sequences of the HLA-DR β 1 chains of these haplotypes revealed that HLA-DR2, -DR5, and -DR8 share the amino acid Asp at position 70, located in the third variable domain, whereas in addition (with the exception of the HLA-DRw11 β chain, expressed by the EBV-LCL JVM), all haplotypes shared the Phe residue at position 67 (Fig. 5). Taken together, these results suggest that the degenerate restriction in the recognition of *Der p* I by these T cell clones could be due to the presence of homologous residues at positions 67 and 70 in the third variable domain of the different HLA-DR β 1 chains.

Stimulation with T cell epitopes present on *Der p* I induces a Th2-like cytokine response by *Der p* I-specific T cell clones. Cytokine-containing supernatants were prepared by stimulation of the NP and AC T cell clones with synthetic peptides corresponding to the respective minimal activating epitopes on the *Der p* I molecule. Since the amount of *Der p* I protein in the *Der p* I deletion mutants could not be accurately determined due to the presence of the glutathione transferase fusion product, experiments were carried out with purified synthetic peptides. As it is shown in Table IV, stimulation of NP T cell clones with the p94–104 peptide at an optimal dose of 1 μ g/ml and the T cell clones AC-15, AC-51, and AC-39 with a 1/5000 dilution of r*Der p* I or p45–67 and p117–143, respectively, resulted in the production of very high levels of IL-4 and IL-5. The levels of the latter two cytokines were increased 10- to 20-fold, as compared to those produced by TT-specific T cell clones obtained from the same patients, whereas low or nondetectable levels of IFN- γ , and low levels of IL-2 were measured. Peptides smaller than the p94–104 minimal epitope recognized by NP-12, NP-14, and NP-44 did not induce any cytokine production (data not shown).

Importantly, the impaired capacity of the *Der p* I-specific T cell clones to produce IL-2 and IFN- γ was not an intrinsic property of these cells. Stimulation by combinations of TPA and calcium ionophore A23187 resulted in the secretion of high levels of these cytokines by all *Der p* I T cells clones tested, simultaneous to their production of IL-4 and IL-5 (Table IV), indicating that the *Der p* I-specific T cell clones are not defective in the production of the Th1 lymphokines IL-2 and IFN- γ .

DISCUSSION

In the present paper, we have analyzed the presence of epitopes on the *Der p* I molecule that are involved in the activation of allergen-specific T cells. To that extent, *E. coli*-expressed recombinant deletion mutants of *Der p* I

were examined for their ability to activate cloned CD4⁺ *Der p* I-specific T cell lines, isolated from the PBMC from two atopic patients strongly allergic to *Der pt* Ag. These *Der p* I-specific T cell clones detected at least three different T cell activation-inducing epitopes, p45–67, p94–104, and p117–143, on *Der p* I, all located within the cysteine-rich N-terminal part of the molecule. Fine mapping of the p45–67 and p117–143 sequences is in progress.

Although the panel of T cell clones used in this study is limited, it seems that no predominant TCR V α or V β gene products are involved in the recognition of the *Der p* I epitopes. For example, none of the three *Der p* I-specific NP T cell clones that recognized the p94–104 sequence share any TCR V α or V β chains. Recently, we have screened a larger panel of *Der p* I-specific T cell clones and confirmed that the recognition of this allergen is not oligoclonally restricted (H. Yssel, R. O'Hehir, and J. Lamb, unpublished observations). In addition, these results confirm the notion that different TCR can recognize the same sequence in the context of the same MHC class II molecule (30).

The epitopes, p45–67 and p117–143, were recognized by the T cell clones from patient AC in the context of HLA-DR7. The recognition of p94–104 by *Der p* I-specific HLA-DR11⁺ (DR5) T cell clones, however, showed a degenerate restriction, in that this epitope could be recognized in the context of HLA-DR2, -DRw11, or -DR8 molecules.

It is firmly established now that the HLA-DR antigens on most haplotypes are encoded by two distinct loci, DR β 1 and DR β 3. Coupling of the products of these loci to HLA-DR α constitutes HLA-DR $\alpha\beta$ 3, which represent the serologically defined major DR- and the supertypic (DRw52) specificities, respectively (31). The latter has been shown to include at least three alleles, DRw52a, DRw52b, and DRw52c (32). Both the major DR-, as well as the supertypic specificities, are able to restrict Ag recognition by T cells (33). For example, O'Hehir et al. (14) have recently demonstrated that HLA-DRw52b can be functional in the recognition of *Der p* I.

Using murine fibroblasts, expressing the DR β 1 encoded DRw11, or the DR β 3 gene products, DRw52a, DRw52b, and DRw52c, the involvement of a DR β 3-encoded molecule in the recognition of *Der p* I by the NP T cell clones was excluded. Comparison of the amino acid sequences of the DR β 1-encoded chains of the HLADR-2, -DRw11, and -DR8 molecules indicated that the degenerate restriction of the recognition of the p94–104 peptide appears to be due to the presence of the Asp(70) and the Phe(67) in

	1	10	20	30	40	50	60	70	80	90
DR1	G D T R P R F L W Q	L K F E C H F F N G	T E R V R L L E R C	I Y N Q E E S V R F	D S D V G E Y R A V	T E L G R P D A E Y	W N S Q K D L L E Q	R R A A V D T Y C R	H N Y G V G E S F T	V Q R R
DR2-Dw2 (PGF) (DRB5-0101)	 Q .	D . Y	 F . H . D	 D L . .			 F . D			
DR2-Dw12 (DRB-0102)	 Q .	D . Y	 F . H . G	 N . .			 F . D			
DR2-Dw21 (AZH) (DRB5-0201)	 C . Q .	D . Y	 F . H . G	 N . .			 I . .	A	 A V	
DR2-Dw22 (REM) (DRB5-0202)	 C . Q .	D . Y	 F . H . G	 N . .			 I . .	A	 A V	
DRw11-Dw5	 E Y	S T S	 F . D . Y F	 Y . .	 F	 E . .	 F . D			
DRw11-JVM	 E Y	S T S	 F . D . Y F	 Y . .	 F	 E . .	 I . .	E	 V	
DRw11-3new	 E Y	S T S	 F . D . Y F	 Y . .	 F	 E . .	 F . D	E	 V	
DRw8-Dw8.1	 E Y	S T G . . Y . .	 F . D . Y F	 Y . .	 S		 F . D	 L	 ? ? ? ? ?	? ? ? ? ?
DRw8-Dw8.2 SPL	 E Y	S T G . . Y . .	 F . D . Y F	 Y . .			 F . D	 G L	 ? ? ? ? ?	? ? ? ? ?

Figure 5. Primary amino acid sequence of the B1 domains of DR1, -2, -5, and -8 MHC class II proteins.

TABLE IV
Cytokine production by *Der p* I-specific T cell clones following Ag-specific or polyclonal activation

Stimulation ^a	IL-2	IL-4	IL-5	IFN- γ
	ng/10 ⁶ cells/ml (mean $\pm$ SD) of triplicate cultures ^b			
NP-12				
p94-104	<0.01	31.1 $\pm$ 10.0	24.3 $\pm$ 0.4	<0.1
TPA + A23187	5.9 $\pm$ 0.7	21.8 $\pm$ 6.3	34.1 $\pm$ 0.6	24.4 $\pm$ 5.2
Con A	<0.01	9.8 $\pm$ 3.6	9.7 $\pm$ 1.2	1.0 $\pm$ 0.7
NP-14				
p94-104	<0.01	17.8 $\pm$ 5.7	15.6 $\pm$ 2.0	<0.1
TPA + A23187	6.7 $\pm$ 0.9	36.0 $\pm$ 7.4	29.8 $\pm$ 3.0	60.0 $\pm$ 7.4
Con A	<0.01	14.0 $\pm$ 2.8	8.6 $\pm$ 0.5	8.9 $\pm$ 0.5
NP-44				
p94-104	<0.01	25.9 $\pm$ 3.0	2.7 $\pm$ 0.2	<0.1
TPA + A23187	24.8 $\pm$ 2.5	40.6 $\pm$ 12.4	3.6 $\pm$ 0.9	47.9 $\pm$ 5.6
Con A	0.6 $\pm$ 0.0	12.1 $\pm$ 2.5	0.6 $\pm$ 0.3	<100
AC-07				
<i>Der p</i> I	<0.01	10.8 $\pm$ 2.1	11.9 $\pm$ 1.8	<0.1
TPA + A23187	43.4 $\pm$ 2.4	15.9 $\pm$ 3.5	13.5 $\pm$ 2.9	>100
Con A	1.9 $\pm$ 0.1	9.6 $\pm$ 2.6	9.6 $\pm$ 2.3	19.6 $\pm$ 2.5
AC-15				
<i>Der p</i> I	<0.01	12.9 $\pm$ 0.2	1.9 $\pm$ 0.2	<0.1
TPA + A23187	5.3 $\pm$ 0.7	32.2 $\pm$ 0.9	3.5 $\pm$ 0.9	>100
Con A	0.3 $\pm$ 0.0	17.2 $\pm$ 1.2	1.6 $\pm$ 0.3	15.5 $\pm$ 0.6
AC-39				
<i>Der p</i> I	<0.01	19.6 $\pm$ 4.6	9.4 $\pm$ 2.2	<0.1
TPA + A23187	5.6 $\pm$ 0.8	20.9 $\pm$ 3.4	13.3 $\pm$ 3.0	41.6 $\pm$ 10.2
Con A	3.4 $\pm$ 0.2	9.9 $\pm$ 5.4	5.6 $\pm$ 1.0	11.3 $\pm$ 7.8
TT-specific clones ^c				
TT	5.9 $\pm$ 3.2	2.4 $\pm$ 1.8	3.4 $\pm$ 1.8	20.8 $\pm$ 16.2
TPA + A23187	7.4 $\pm$ 5.2	4.5 $\pm$ 2.5	3.9 $\pm$ 2.7	30.5 $\pm$ 26.8

^a One million T cells were stimulated with 1 μ g/ml of the synthetic *Der p* I peptide p94-104 (NP) or with 1/5000 dilution of rec *Der p* I (AC) or with 1 μ g/ml of TT. Concentrations of TPA, A23187, and Con A, 1 ng/ml, 500 ng/ml, and 20 μ g/ml, respectively.

^b Cytokines were measured in 24-h culture supernatants by specific ELISA.

^c Results of 8 TT-specific T cell clones derived from patients NP and AC.

the three β chains. Variations in the first and second hypervariable regions of the HLA-DR β chain cannot account for this finding. Substitution of the Phe(67) by Ile(67), as is the case for the EBV-LCL JVM, resulted in the inability of the APC to present the peptide, suggesting that Phe(67) and Asp(70) are important for recognition of the p94-104 sequence by the *Der p* I-specific T cell clones. This notion is supported by the observation that p94-104 pulsed EBV-LCL REM, and AZH, which both have an isoleucine on position 76 and a glutamine at position 70, were unable to induce proliferative responses in the T cell clones NP-12, NP-14, and NP-44. It has to be noted here that JVM, REM, and AZH can effectively present peptides and induce proliferation of TT-specific T cells, indicating that these EBV-LCL are able to function as an APC.

Since peptides are recognized by CD4⁺ T cells in the context of MHC class II molecules, analysis of T cell clones from donors with different HLA haplotypes is likely to result in the demonstration of additional T cell activation epitopes on the *Der p* I molecule. However, the finding that the same *Der p* I peptides can be recognized in the context of different class II MHC molecules suggests that the number of epitopes on the *Der p* I molecule may be rather limited, although additional studies using T cell clones derived from patients with different haplotypes will be required.

The recognition of the *Der p* I T cell epitopes by the *Der p* I-specific T cell clones correlated well with the production of several cytokines. Interestingly, stimulation of the T cells with the relevant peptides resulted in the production of unusually high levels of IL-4 and IL-5, but of low

to nondetectable levels of IL-2 and IFN- γ . Although the vast majority of CD4⁺ T cell clones obtained from healthy donors simultaneously produce IL-2, IL-4, IL-5, and IFN- γ upon activation (34, 35), it is now clear that in certain pathologic conditions Th cells may show shifts in cytokine production profiles. T cell clones specific for bacterial antigens (36-39) on the one hand, and allergen-specific T cell clones (38-40) on the other hand have been described to exhibit Th1- or Th2-like production profiles, respectively, reminiscent of those described for mouse Th cells (41). Therefore, it seems that chronic stimulation with *Der p* I may select for *Der p* I-specific T cell clones with a Th2-like type of cytokine production in atopic individuals. It should be noted, however, that the cytokine production profiles of the *Der p* I-specific T cell clones described in this study were characterized by unusual high levels of IL-4 and IL-5: approximately 10- to 20-fold higher than those obtained with TT-specific T cell clones from these patients, as well as from healthy individuals and patients suffering from chronic Lyme arthritis or lepromatous leprosy (36, 37). However, some *Der p* I-specific T cell clones produced low or nondetectable amounts of IL-2 and IFN- γ , following allergen-specific stimulation. Taken together, these observations indicate that the classification of human T cells into different subsets should be based upon differences in the ratio of the production of various cytokines, rather than their inability to produce certain cytokines. This notion is supported by the finding that the failure to produce normal levels of IL-2 and IFN- γ was not due to suboptimal activation with Ag and was not an intrinsic property of the *Der p* I-specific T cells. Stimulation of the *Der p* I-specific T cell clones with the optimal concentrations of combinations of TPA and the calcium ionophore A23187 resulted in high production levels of all four cytokines, including IL-2 and IFN- γ , whereas stimulation with Con A represented more the situation following Ag-specific stimulation. These results suggest that activation of *Der p* I-specific T cell clones, via pathways which circumvent triggering of the TCR/CD3 complex, result in cytokine production patterns that are different from those obtained after allergen-specific stimulation. Although such stimuli may be not representative of the natural situation, they indicate that *Der p* I specific T cells with a restricted cytokine production profile are not defective in their capacity to produce the Th1 lymphokines IL-2 and IFN- γ .

Among the various cytokines, IL-4, IL-5, and IFN- γ in particular have been found to be involved in the regulation of the IgE response. IL-4 induces IgE switching and IgE synthesis. This IL-4-induced IgE synthesis is enhanced by IL-5, whereas IFN- γ has strong inhibitory effects (42-44). Interestingly, the *Der p* I-specific T cell clones described in this study were found to induce the synthesis of significant levels of IgE by purified B cells in vitro, in the absence of exogenously added IL-4⁴. Given the central role of T cells in the regulation of the IgE response, the use of modified synthetic peptides covering the epitopes recognized by these T cell clones to block the recognition of total *Der p* I Ag or interfere with activation of the T cells, may be important in the abrogation or

⁴ Yssel, H., G. G. Aversa, H. Gascan, A. Terr, and Jan E. de Vries. Induction of anergy by *Der p* I derived peptides in T cell clones specific for *Dermatophagoides pteronyssinus*. Submitted for publication.

down regulation of the synthesis of IgE. In this respect, it is of importance to notice that none of the *Der p 1* peptides was recognized by a pool of antisera obtained from ten different atopic patients allergic to *Der pt*, as revealed by Western blotting (K. Johnson and H. Yssel, unpublished observations). This indicates that purified *Der p 1* peptides may be used therapeutically or diagnostically without the risk of provoking anaphylactic reactions. Studies are in progress to determine whether such modified peptides can block activation of T cells and subsequent IgE production in vitro.

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