

The *Dermatophagoides pteronyssinus* Group 2 Allergen Contains a Universally Immunogenic T Cell Epitope

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The use of T cell epitope-containing peptides for the induction of anergy in allergen sensitization is limited by genetic restriction that could be circumvented by using universally immunogenic epitopes. We attempted to identify such epitopes on *Dermatophagoides pteronyssinus* group 2 allergen (Der p 2), a major allergen of *D. pteronyssinus* T cells from BALB/c (H-2^d), C57BL/6 (H-2^b), C3H (H-2^k), and SJL (H-2^s) mice that were immunized with rDer p 2, recognized an immunodominant region encompassing residues 21–35. A synthetic 21–35 peptide (p21–35) induced strong dose-dependent in vitro T cell proliferation with cells of the four mouse strains and required processing for MHC class II presentation. Substitution of Ile²⁸ with Ala resulted in reduction of T cell proliferation in each strain. Ile²⁸ could represent an important MHC class II anchoring residue for T cell response to p21–35. An immunodominant T cell epitope of Der p 2 therefore behaves as a universal epitope and could be a suitable candidate for T cell anergy induction. *The Journal of Immunology*, 2002, 169: 2430–2435.

The design of novel forms of immunotherapy for allergic diseases remains a challenge, despite our better understanding of mechanisms by which allergen sensitization occurs. Among several possibilities for intervention, specific T cells have recently attracted much attention. T cells play an important role in the immunopathogenesis of allergic reactions, not only because the production of IgE Abs is strictly T cell dependent, but also because T cells produce soluble factors such as cytokines, which attract other cells participating in allergic inflammation. Recent advances regarding specific immunotherapy have indeed underscored the potential for T cell epitope-containing peptides to induce anergy (1). Thus, immunodominant T cell epitopes from a number of allergens have been used in clinical trials with significant benefit (2, 3), suggesting that elucidating the mechanism of action of such epitopes could improve therapeutic strategies.

However, most defined T cell-stimulating regions have limited activity across divergent MHC class II haplotypes, i.e., different animal strains preserve and present different Ag regions for stimulation of T cells. This genetically restricted T cell stimulatory activity of peptides has been a serious obstacle to the development of synthetic vaccines, which practically should be effective in genetically diverse populations. Recently, however, T cell-stimulating peptides were described that are active across multiple mouse haplotypes and/or in association with most human class II MHC DR molecules (4–7), thereby providing a significant step forward in the design of universally active T cell activators. A universal T cell epitope refers to a Th peptide that is recognized by MHC class II molecules of almost all animal strains. Such T cell epitopes could be used either to induce T cell anergy directly or to provide help to B cells for the production of specific Abs to weak immunogens and, in particular, to peptides (6, 8, 9). Unfortunately, not all universal T cell epitopes are suitable for therapy. For therapeutic

efficacy, such epitopes must also dominate the response to a protein Ag in a given animal species, a property referred to as immunodominance (10). Putative promiscuous T cell epitopes have already been defined in a few allergens, but, with the possible exception of bee venom phospholipase (11), these are not immunodominant, because only a fraction of sensitized individuals recognize such epitopes (12).

The therapeutic potential of peptide-containing T cell epitopes, which would be both immunodominant and universal, prompted us to investigate the immune properties of *Dermatophagoides pteronyssinus* group 2 allergen (Der p 2),² a major allergen from the house dust mite *D. pteronyssinus*. T cell epitopes of Der p 2 have been described in animals (13) and humans (14, 15), but data vary widely because recognition is restricted by different MHC class II haplotypes or different HLA molecules (13, 16). In this report, we describe the properties of a major T cell epitope, which is presented in a seemingly MHC-permissive manner.

Materials and Methods

Synthetic peptides

Peptides, synthesized by Eurogentec (Seraing, Belgium) and shown in Table I, include the following: 1) 15-mer peptides encompassing the entire Der p 2 sequence with 5-aa overlaps, 2) 12-mer peptides covering residues 10–39 of Der p 2 with an 11-aa overlap, 3) 15-mer peptides covering residues 21–35 of Der p 2, with each residue substituted by Ala, and 4) a 15-mer peptide containing residues 830–844 of tetanus toxoid.

Recombinant Der p 2

The coding sequence for full-length Der p 2 was produced by RT-PCR and inserted into the pPICZαC expression vector (Invitrogen, Leek, The Netherlands). Positive clones were confirmed by PCR using Der p 2 specific primers (17) and were further verified by DNA sequencing. Plasmid cDNA was purified and transformed into *Pichia pastoris* using the Easy Select *Pichia* Expression kit (Invitrogen). Cells were first cultured in buffered minimal glycerol medium and transferred into minimal methanol medium containing methanol for induction of protein expression (18). Recombinant Der p 2 was purified on a Superdex 75 column (Pharmacia Biotech, Uppsala, Sweden) and was assessed by SDS-PAGE. Protein concentration was determined by light absorption at 280 nm. Correct folding of rDer p 2 was evaluated using conformation-dependent specific mAbs produced in our laboratory.

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² Abbreviations used in this paper: Der p 2, *Dermatophagoides pteronyssinus* group 2 allergen; LN, lymph node; P, position.

Table I. Peptide sequences

Peptide	Position	Sequence
p1	1–15	DQVDVKDCANHEIKK
p11	11–25	HEIKKVLVPGCHGSE
p21	21–35	CHGSEPCIIHRGKPF
p31	31–45	RGKPFQLEAVFEANQ
p41	41–55	FEANQNTKTAKIEIK
p51	51–65	KIEIKASIDGLEVDV
p61	61–75	LEVDVPGIDPNACHY
p71	71–85	NACHYMKCPLVKGQQ
p81	81–95	VKGQQYDIKYTNVNP
p91	91–105	TWNVPKIAPKSKNVV
p101	101–115	SENVVVTVKVMGDDG
p111	111–129	MGDDGVLACAIATHAKIRD
TT	830–844	QYIKANSKFIGITEL
m10	10–21	NHEIKKVLVPGC
m11	11–22	HEIKKVLVPGCH
m12	12–23	EIKKVLVPGCHG
m13	13–24	IKKVLVPGCHGS
m14	14–25	KKVLVPGCHGSE
m15	15–26	KVLVPGCHGSEP
m16	16–27	VLVPGCHGSEPC
m17	17–28	LVPCHGSEPCI
m18	18–29	VPCHGSEPCII
m19	19–30	PGCHGSEPCII
m20	20–31	GCHGSEPCIIHR
m21	21–32	CHGSEPCIIHRG
m22	22–33	HGSEPCIIHRGK
m23	23–34	GSKPCIIHRGKP
m24	24–35	SEPCIIHRGKPF
m25	25–36	EPCIIHRGKPFQ
m26	26–37	PCIIHRGKPFQL
m27	27–38	CIIHRGKPPQLE
m28	28–39	IIHRGKPFQLEA

Animals and immunization

BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from the University of Leuven animal facilities. C3H (H-2^k) and SJL (H-2^s) were purchased from Harlan (Zeist, The Netherlands). Mice were immunized when 6–8 wk old by s.c. footpad injection of 100 μ l containing 50 μ g/ml peptide or 10 μ g/ml rDer p 2 emulsified in CFA, followed by a second injection at day 14 with the same dose of Ags in IFA. All animal experiments were conducted in accordance with the regulations of the University of Leuven Review Board.

APCs

Splenocytes from syngeneic naive animals were used for Ag presentation after irradiation (2500 rad). APCs were added to 96-well culture plates at a concentration of 4×10^5 /well and were incubated for 4–5 h at 37°C with 30 μ g/ml test Ag before addition of T cells. This concentration was selected because preliminary experiments with 0.3, 3, and 30 μ g/ml peptide or rDer p 2 showed that 30 μ g/ml yielded the most reproducible results. A total of 5×10^6 nonirradiated cells per milliliter were fixed with 0.05% glutaraldehyde in PBS for 30 s at room temperature, after which an equal volume of 0.2 M lysine in PBS, pH 7.4, was added. The cells were then centrifuged and washed twice before use. Ag processing by APCs was blocked by treatment with the lysosomotropic agent chloroquine. Thus, 10 mM chloroquine (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5×10^{-5} M 2-ME, and 5% heat-inactivated FCS was filtered and stored at –20°C for subsequent use. Irradiated naive spleen cells were incubated in culture medium containing 40 μ M chloroquine for 15 min at 37°C before addition of Ag. Four hours later, APCs were washed twice with RPMI 1640, and their capacity to activate T cells was tested as described below.

Preparation of T cells and proliferation assays

Ten days after the last immunization, popliteal lymph nodes (LNs) were removed and T cell-enriched fractions were purified by negative selection using Abs against CD45R, CD11b, and CD11c coupled to magnetic beads (MACS System; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of T cell suspensions by FACS analysis was $\geq 97\%$. Purified T cell

suspensions were adjusted to a concentration of 4×10^6 cells/ml in RPMI 1640, and 100 μ l were cultured in 100 μ l of RPMI 1640 containing the test Ag and irradiated syngeneic naive spleen cells. After 4 days, the cells were pulsed with [³H]thymidine for an additional 18 h. All tests were performed in triplicate. Proliferative responses are expressed as Δ cpm $\times 10^{-3}$ after subtracting background levels in the absence of Ag, which varied between 800 and 1000 cpm.

Cytokine assays

Cytokine concentrations were measured in supernatants of cell cultures obtained after 48 or 72 h for IL-4 and IFN- γ , respectively. The sensitivity of the ELISAs (Biosource, Nivelles, Belgium) was 1 pg/ml for IFN- γ and 2.5 pg/ml for IL-4.

Results and Discussion

p21–35 is recognized in the context of different MHC class II haplotypes

We have previously shown that BALB/c mice (H-2^d) produce high titers of specific Abs upon immunization with Der p 2 and that a T cell epitope is located between residues 21 and 35 (19). To determine whether the T cell epitope is dominant, T cells were prepared from LN cells of six BALB/c mice primed by s.c. footpad injection of rDer p 2 and were pooled and restimulated in vitro with a series of 15-mer overlapping peptides spanning the entire Der p 2 sequence. T cells responded almost exclusively to two epitopes contained within residues 21–35 and 71–85, respectively (Fig. 1A). Only minor, nonsignificant T cell proliferative responses were observed with the remaining peptides. The proliferative response of T cells was completely blocked by addition of anti-I-Ek and I-Ab mAb, which cross-react with cells of the H-2^d haplotype,

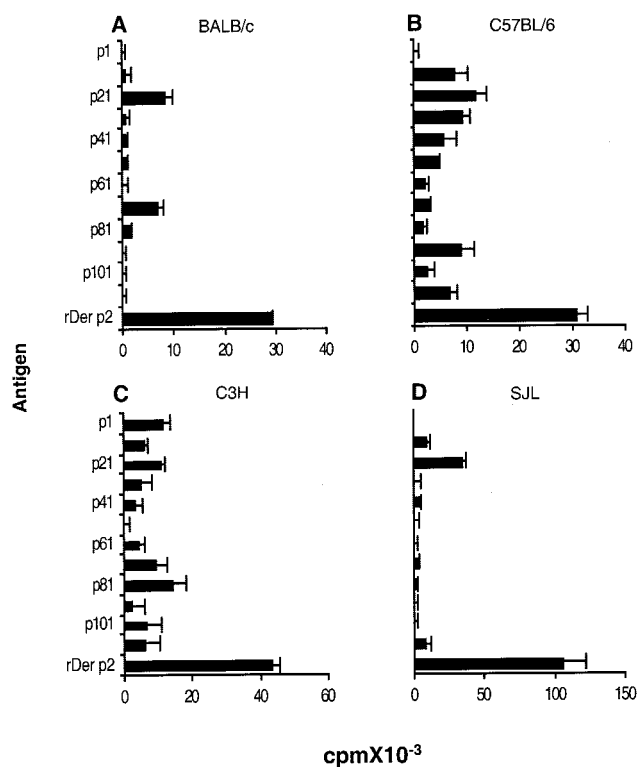


FIGURE 1. Proliferation of T cells from rDer p 2-immunized mice of four different haplotypes. T cells were challenged in vitro with 12 Der p 2-derived peptides (30 μ g/ml, 13 μ M) covering the full length of Der p 2 with a 5-aa overlap or with rDer p 2 (30 μ g/ml, 2 μ M). Values are expressed as mean cpm $\times 10^{-3} \pm$ SD of triplicates after subtracting background values. The experiment was conducted twice with similar results.

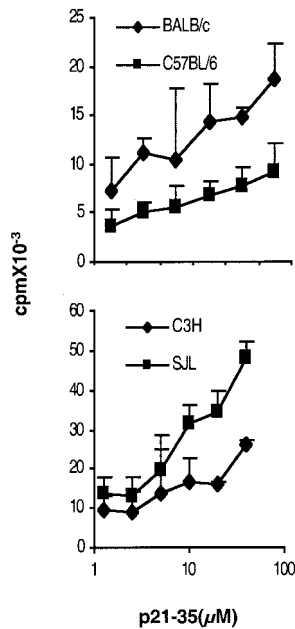


FIGURE 2. Proliferation of T cells from p21-35-immunized mice expressing four different haplotypes. Purified T cells from LNs of each mouse strain were stimulated with different concentrations of p21-35. Results are expressed as mean $\text{cpm} \times 10^{-3} \pm \text{SD}$ of triplicates after subtracting background values. The experiment was performed twice yielding similar results.

thereby confirming that proliferation of T cells is dependent on binding of the peptide to MHC class II molecules (data not shown). There is considerable mouse strain-dependent variability in allergen-induced Ig responses—particularly with respect to IgE and IgG1 (20). Notably, and in contrast to our findings, H-2^d mice reportedly respond poorly to Der p 2 immunization (13, 21). Therefore, LN cells were obtained from three additional Der p 2-immunized mouse strains expressing different MHC class II haplotypes, including the C57BL/6 (H-2^b). As shown in Fig. 1, B–D, a significant in vitro T cell response to p21-35 was obtained in each case. In addition, the highest levels of specific anti-Der p 2 Abs, including IgG1, were observed in H-2^d mice (data not

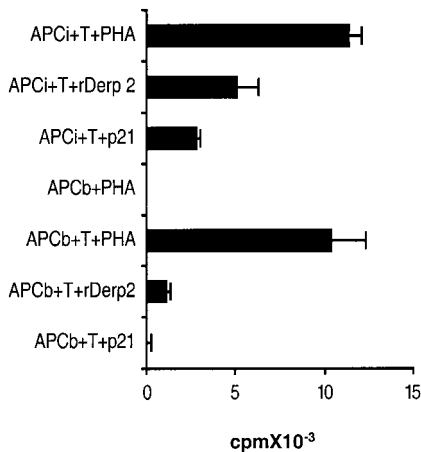


FIGURE 3. T cell proliferation with blocked or irradiated APCs. T cells from p21-35-immunized BALB/c mice were stimulated with p21-35 (30 $\mu\text{g}/\text{ml}$, 13 μM) in vitro in the presence of chloroquine-blocked or irradiated syngeneic APCs. Values are expressed as $\text{cpm} \times 10^{-3} \pm \text{SD}$ of triplicates after subtracting background values. APCb, Chloroquine-blocked APC; APCi, irradiated APC.

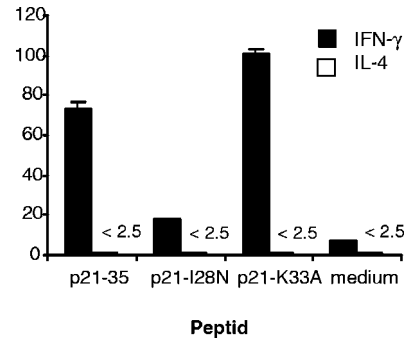


FIGURE 4. Cytokine assay of T cells from p21-35-immunized mice. T cells ($4 \times 10^5/\text{well}$) were incubated with p21-35, mutant peptides (30 $\mu\text{g}/\text{ml}$, 13 μM), or medium, and irradiated syngeneic splenocytes. Culture supernatants were assayed by ELISA for IFN- γ and IL-4. Values are expressed as picograms per milliliter $\pm \text{SD}$. The assay was performed twice with similar results.

shown), confirming that the BALB/c strain of mice was suitable to evaluate the anti-Der p 2 responses, at least under the present experimental conditions.

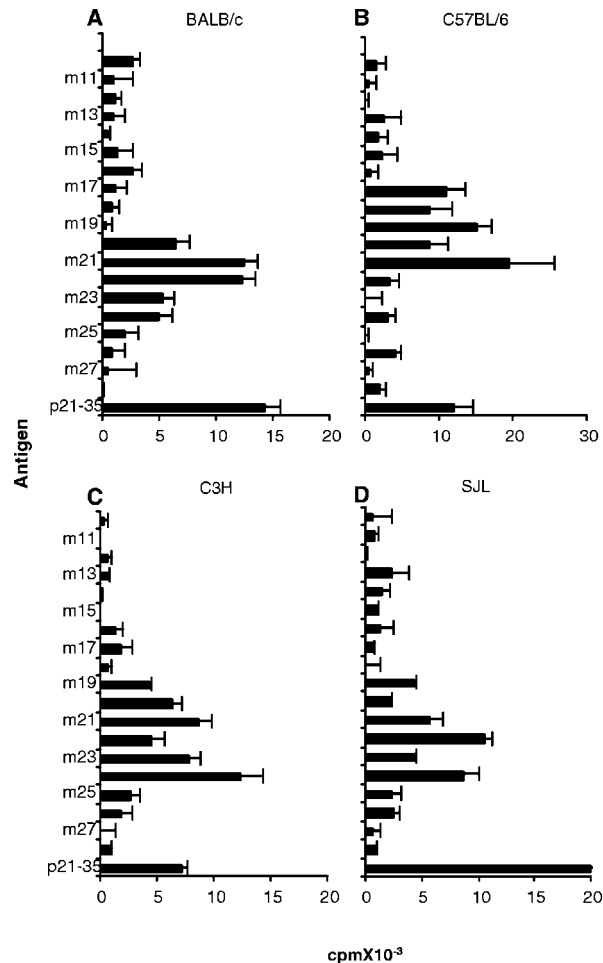


FIGURE 5. Determination of the core T cell epitope of p21-35-immunized mice of four different haplotypes. T cells were challenged in vitro with a panel of 12-mer synthetic peptides (30 $\mu\text{g}/\text{ml}$, 16 μM) covering Der p 2 residues 10-39, with 1 aa offset from the previous peptide. Values are expressed as mean $\text{cpm} \times 10^{-3} \pm \text{SD}$ of triplicates after subtracting background values. The experiment was performed twice with similar results.

To confirm these findings, six mice of each MHC haplotype were immunized with p21–35. The T cells were collected and purified as described above. Significant dose-dependent T cell-proliferative responses to both p21–35 and rDer p 2 (data not shown) were now observed in all cases (Fig. 2). These results demonstrate that p21–35 exhibits the structural characteristics of a universal T cell epitope, which can be recognized by T cells in the context of at least four different class II MHC-encoded alleles. Interestingly, a search for sequence homology identified FIS, a peptide from sperm whale myoglobin encompassing residues 106–118 (FISEAIIHVLHSR), as having the sequence SE-X (X)-IIH in common with p21–35 (CHGSEPCIIHRGKPF). FIS contains a Th cell epitope for restriction elements H-2^d, H-2^k (22), H-2^s (23), and H-2^b (data not shown), and as such is considered to be universal (8).

Results from our laboratory (V. Carlier, B. Wu, W. Janssens, M. Jacquemin, and J. M. R. Saint-Remy, manuscript in preparation) show that p21–35 induces significant proliferation of T cells obtained from PBMCs of Der p 2-sensitive patients, suggesting that the present data can be extrapolated to human allergic individuals. Recognition of p21–35 by human T cells is in keeping with data provided by O’Hehir et al. (14), showing that six of nine sensitive individuals reacted to p11–50, but not with the data of O’Brien et al. (15). This apparent inconsistency may be due to a Der p 2 polymorphism, as previously discussed. Furthermore, as the p21–35 T cell epitope is located within a conserved region of the molecule (24), it is likely that conclusions drawn from the study of Der p 2 can be extended to at least some of the other members of group 2 allergens, such as Der f 2 and Der m 2.

p21–35 presentation requires processing by APCs

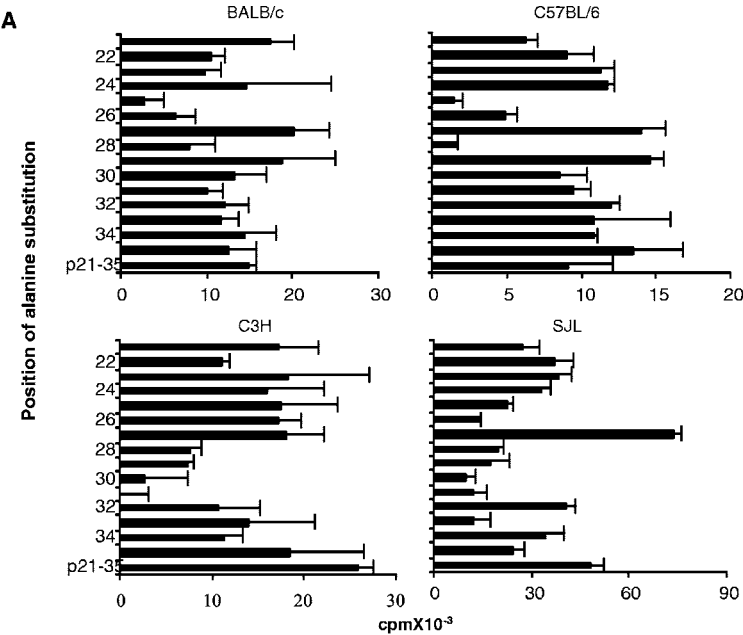
A majority of the short peptides described so far do not require processing (25, 26), unless they contain one or more cysteines

(27). In our study, p21–35 contains two such cysteines. The strong T cell-activating properties of p21–35 observed in the context of several MHC class II haplotypes prompted us to verify whether p21–35 required processing by APCs. Thus, spleen cells from naive mice were treated with glutaraldehyde or were irradiated before incubation with either p21–35 or PHA. The cells were then incubated with purified T cells from LNs of p21–35 immunized BALB/c mice. The results suggested that p21–35 required cell processing for specific T cell activation, because its stimulatory effect persisted in the presence of irradiated APCs, but not in the presence of glutaraldehyde-fixed APCs (data not shown). However, because MHC molecules could have been altered by glutaraldehyde treatment, assays were also conducted using chloroquine-treated APCs. Data shown in Fig. 3 confirm that p21–35 required processing for effective presentation by MHC class II determinants.

p21–35 elicits Th1-like cytokines in vitro

We next evaluated the cytokine secretion profile of p21–35-activated T cells. In vitro stimulation of purified T cells isolated from regional LNs of p21–35 footpad-immunized BALB/c mice resulted in high levels of IFN- γ , but undetectable levels of IL-4 and IL-10 (data not shown), suggesting a Th1 subset phenotype (Fig. 4). The same Th1-like cytokine profile was observed after p21–35 immunization of H-2^b, H-2^k, and H-2^s mice. We have previously shown that substitution of Ile²⁸ by Ala or Asn abolishes p21–35-induced T cell proliferation (19), and we now demonstrate that this is accompanied by a significant reduction in IFN- γ production. The latter was not affected by the Lys³³Ala substitution, which eliminates the major p21–35 B cell epitope (19). The production of cytokines by polyclonal T cells toward p21–35, however, could have been biased by the use of CFA (28).

FIGURE 6. Determination of p21–35 amino acid residues relevant to T cell activation of p21–35-immunized mice of four different haplotypes. T cells were challenged in vitro with a panel of 15-mer synthetic peptides (30 μ g/ml, 13 μ M) covering Der p 2 residues 21–35, with 1 aa substituted by Ala. Values are expressed as mean cpm $\times 10^{-3} \pm$ SD of triplicates after subtracting background values (A). The critical residues for T cell response to p21–35 are summarized in B. A value which is at least 2-fold lower than that obtained with the wild-type sequence is considered as significant (*).



Mice strain	Haplotype	T epitope	Position of alanine substitution										
			21	22	23	24	25	26	27	28	29	30	31
			C	H	G	S	E	P	C	I	I	H	R
BALB/c	H-2 ^d	24-31					*	*		*			
C57BL/6	H-2 ^b	21-28					*	*		*			
C3H	H-2 ^k	24-30								*	*	*	
SJL	H-2 ^k	24-30					*	*		*	*	*	

Three T cell epitopes are identified within p21–35 in different MHC class II haplotypes

The observation that p21–35 contains an immunodominant universal T cell epitope led us to further analyze the structural characteristics of the epitope. Two types of universal T cell epitopes have been identified (29). In the first, a single epitope anchors to different MHC class II alleles. In the second, several overlapping epitopes are present that can accommodate different MHC class II alleles. To determine which type of T cell epitope was recognized in the context of the four different MHC alleles used here, purified T cells obtained from p21–35 immunized mice were cultured in the presence of a panel of 12-mer synthetic peptides covering Der p 2 residues 10–39. As shown in Fig. 5, significant proliferative responses were obtained to residues 24–31 for BALB/c, 21–28 for C57BL/6, and 24–30 for C3H and SJL mice, respectively. Although the immunodominant epitopes recognized in the context of the different haplotypes overlap by only a few a residues, they are clearly distinct. Therefore, at least three different T cell epitopes appear to be located within the region encompassing residues 21–31. A T cell line produced from BALB/c mice immunized with p21–35 similarly proliferated when exposed to residues 24–31 (data not shown).

Ile²⁸ is critical for T cell responses in different MHC haplotypes

The binding of a peptide to MHC molecules depends on only a few residues, referred to as positions 1 (P1), 4 (P4), 6 (P6), and 9 (P9). Because the P1 residue appears to be degenerate, peptide specificity and binding capacity depend on P4 and P6. The contribution of single amino acid residues for either MHC class II anchoring or TCR recognition was determined using Ala scanning, in which each p21–35 amino acid residue was substituted in turn by Ala. Several critical residues in each MHC haplotype were identified by proliferation assays performed with T cells from p21–35 immunized mice (Fig. 6A). Importantly, substitution of Ile²⁸ significantly reduced T cell responses obtained with p21–35 in all four murine haplotypes (Fig. 6B). In a previous study, Ile²⁸ had been identified using known algorithms as a putative P4 anchoring residue in H-2^d haplotype (19, 30). Because the T cell epitopes defined for H-2^k and H-2^s mice are almost identical (Fig. 5), Ile²⁸ can be considered the P4 anchoring residue of p21–35 in these two MHC haplotypes. In H-2^b, the T cell epitope is located at the N terminus of the peptide (Fig. 5), in which Pro²⁶ is the putative P4 anchoring residue, because Pro shares attributes with those of Ile (31).

Comparable reductions in T cell proliferative response were observed in the four haplotypes when using an Ile²⁸Asn mutant peptide instead of Ile²⁸Ala (data not shown), suggesting that the reduction is position dependent. Ile²⁸, therefore, might be a critical residue for MHC class II anchoring of p21–35. Flanking residues influence peptide T cell activation properties (32). Significant reductions in T cell response were observed for some haplotypes, particularly H-2^k mice, in which residue Arg³¹ completely abrogated the T cell response (Fig. 6A).

An interesting observation made in the present study is that Ala substitution of Cys²⁷ increases T cell proliferation, as compared with the results obtained with the immunizing peptide. This effect might be mediated by peptide cysteinylolation, as has been reported (33). The second Cys is located at residue 21, well outside of the actual T cell epitope. This increased T cell proliferation to the Cys²⁷Ala p21–35 mutant could have an impact on the capacity to induce tolerance by administration of p21–35.

Therefore, we have identified a T cell epitope expressed on a major allergen that is both immunodominant and universal. Such an epitope could be an ideal candidate for induction of anergy to

Der p 2. The fact that it is located in a conserved region of the Der p 2 molecule offers the further advantage of possibly modulating the immune response toward alternative Der p 2 isoforms or other group 2 allergens. It remains to be established whether injection of p21–35 induces linked suppression of the immune response toward Der p 2, as observed in studies with epitopes from other allergens such as Der p 1 (34), and whether both B and T cell epitopes are required for optimal effect of specific allergen vaccination. This would obviously exert a direct influence on the design of therapies using single or multiple epitopes of Der p 2. Experiments performed with p21–35 indicate that linked suppression could require both B and T epitopes (B. Wu, G. Toussaint, W. Janssens, M. Jacquemin, and J. M. R. Saint-Remy, manuscript in preparation). Finally, it remains to be determined whether induction of anergy to one major allergen would be sufficient to alleviate the symptoms of patients sensitized to multiple components of house dust mite.

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