

Major T cell epitope-containing peptides can elicit strong antibody responses

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Peptides containing major T cell epitopes have the capacity to induce T cell anergy and have therefore been proposed for the treatment of allergic and autoimmune diseases. Such peptides should not be immunogenic, *i.e.* should not contain a B cell recognition site. We have evaluated in BALB/c mice the therapeutic potential of a 15-mer peptide (p21–35) derived from *Der p2*, a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, which contains a dominant T cell epitope but is not recognized by antibodies to *Der p2*. Unexpectedly, p21–35 elicited strong immune responses, suggesting the presence of a cryptic B cell epitope. Similar results were obtained with mice of three additional MHC haplotypes. A core sequence of four amino acids (Ile-Ile-His-Arg) corresponding to residues 28–31 was shared by the B and T cell epitopes. Critical residues for B cell recognition were Arg31 and Lys33, while Ile28 was essential for T cell recognition. A Lys33Ala mutant of p21–35 still activated T cells but had much reduced immunogenic properties, making it a suitable alternative peptide for T cell anergy induction. Careful investigation of the immunogenic potential of peptides used to induce T cell anergy should be carried out prior to their clinical application.

Key words: Allergen peptide / *Der p2* / Immunotherapy / T cell epitope / B cell epitope

Received	16/7/99
Revised	6/10/99
Accepted	7/10/99

1 Introduction

Administration of T cell epitope-containing peptides has been proposed for the treatment of IgE-mediated allergic diseases and some autoimmune diseases [1]. As the production of high-affinity Ab, including IgE, is T cell dependent, it was suggested that a strategy through which specific T cells would be anergized instead of activated would be of therapeutic value.

CD4⁺ T cells recognize specific peptides presented in the context of MHC class II determinants. T cell activation, however, requires the expression by presenting cells of a number of co-stimulatory molecules, including CD80/87 and CD40 [2]. In the absence of such co-stimulation, T cells are rendered functionally inoperative, or anergized, often after a short period of activation [3]. The mechanisms by which T cells are anergized are the matter of intensive studies [4], yet incompletely understood. Nev-

ertheless, based on results of animal experiments, a number of peptides have already been designed which contain single or combinatory T cell epitope(s) representative of immunodominant T cell epitopes [5–7].

For the therapy of allergic diseases, targeting directly allergen-specific T cell functions was therefore an attractive possibility. This could be accomplished by using allergen-specific peptides that contain functional T cell epitopes but lack IgE binding capacity [8], and indeed a number of clinical trials have been carried out with peptides derived from major allergens of cat or ragweed [9, 10].

The concept is valid insofar as the peptide is not immunogenic *per se*, namely it does not contain a binding region for B cells (Ab). B and T cell epitopes are usually separated on a protein, although often adjacent, which is deemed necessary for an efficient T-B collaboration [11]. A few exceptions to this paradigm have, however, been described, in which epitope contingency or partial overlap was found [12], without obvious hampering of T-B cooperation. B and T cell epitopes have different structural requirements: B cell epitopes are often discontinu-

[1 19897]

Abbreviation: *Der p2*: *Dermatophagoides pteronyssinus* group II

ous [13], which contrasts with the continuous nature of T cell epitopes. This applies in particular to IgE Ab, the epitopes of which are in general highly conformation dependent [14]. T cell epitopes in most cases are distinct from IgE Ab binding domains within an allergen [15].

However, an actual separation of B and T epitopes might not always occur. Studies carried out in allergic diseases with T cell epitope-containing peptides have shown that Ab, including IgE, can be induced which recognize the peptide immunogen [16], with the risk of triggering anaphylactic reactions. The length of the peptide used in such studies was apparently sufficient to form a novel epitope for Ab (IgE or IgG). There are, however, other reasons for which the presence of a B cell epitope on such a peptide can be overlooked. The B epitope can be cryptic, namely not be recognized by Ab elicited towards the full-length parent antigen. Ab generated towards cryptic epitopes may in turn not recognize the antigen and therefore escape detection with native allergen. The design of a T cell epitope-containing peptide usually includes addition of amino acids, in particular whenever several epitopes are linked together, which can generate new binding moieties for B cells. Whatever the case, any peptide contemplated for use in induction of T cell anergy should be carefully checked for its capacity to bind Ab and/or elicit an Ab response.

We have investigated in a mouse model the therapeutic potential of a major T cell epitope of *Der p2* [17, 18], one of the main allergens for the house dust mite *Dermatophagoides pteronyssinus*, for its use in IgE-mediated allergic diseases. However, we soon realized that the selected peptide was immunogenic. We therefore analyzed the basis of this immunogenicity to determine whether it was possible or not to use mutant peptides with T cell-anergizing properties only.

2 Results

2.1 p21–35 contains a major T cell epitope

BALB/c mice ($n = 5$) were immunized by footpad injections of 10 μ g *rDer p2* emulsified in CFA and boosted 2 weeks after with the same dose in IFA. T cells from draining regional LN were purified and used for *in vitro* proliferation assay. Significant proliferation was obtained with both *rDer p2* and p21–35 (data not shown), indicating that the p21–35 region contained a major T cell epitope. To ensure that p21–35 contained the full-length sequence required for efficient T cell activation, BALB/c mice ($n = 5$) were immunized with 50 μ g/ml p21–35 alone. Results presented in Fig. 1 show that T cells proliferate with p21–35 in a dose-dependent manner, as

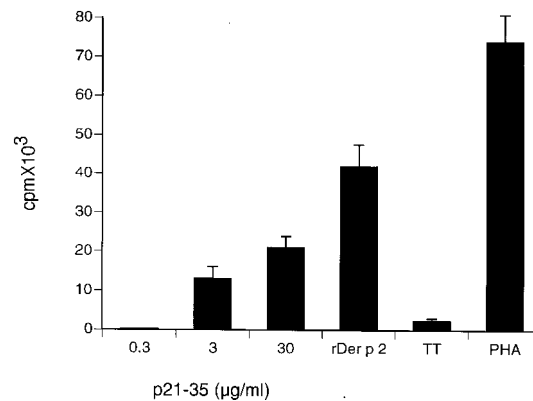


Fig. 1. T cell proliferative response of p21–35-immunized BALB/c mice. T cells obtained from five mice were pooled and tested for *in vitro* reactivity against different concentrations of p21–35, as indicated. Peptide from tetanus toxoid (TT, amino acid 830–844, 30 μ g/ml) and PHA (10 μ g/ml) were included as negative and positive controls, respectively. Results are expressed as mean ($\pm$ SD) of triplicates after subtracting the mean cpm obtained with wells without peptide. The experiments were repeated twice with similar results.

well as with *rDer p2*. This indicated that the T cell epitope on p21–35 is readily accessible and fully efficient to trigger an anti-*rDer p2* immune response.

2.2 p21–35 elicits the production of specific Ab

However, it was soon realized that immunization with p21–35 also elicited a specific Ab production. As shown in Fig. 2, mouse serum recognizes the immunizing peptide but not the two adjacent peptides which share five amino acids each with p21–35, suggesting that critical residues for Ab binding were located within the middle region of p21–35. Interestingly, however, no reactivity was observed with full-length *rDer p2*, suggesting that the B cell epitope was cryptic.

2.3 p21–35 triggers T and B cell responses in mice with different MHC haplotypes

The relevance of these findings to the possibility of using p21–35 for immunotherapy was evaluated by determining whether p21–35 was recognized in the context of other MHC haplotypes. Thus, C57BL/6 (H-2^b), C3H (H-2^k) and SJL (H-2^s) mice were immunized with p21–35 and the presence of specific T cells and Ab was checked by proliferation and antigen-binding assays, respectively. The results shown in Fig. 3A and B demonstrate that the T cell epitope of p21–35 is recognized by the three mouse strains and that a cryptic B cell epitope is also present.

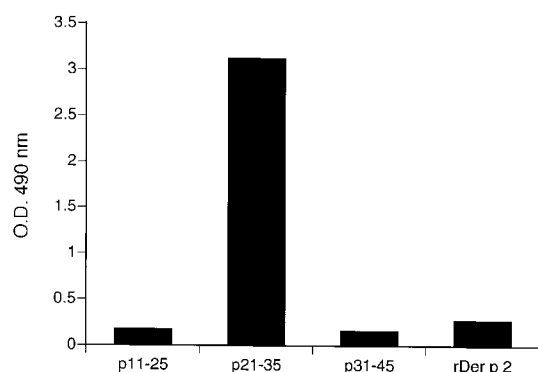


Fig. 2. Binding of anti-p21-35 antisera to p21-35 and the two adjacent peptides, p11-25 and p31-45, or *rDer p2*. A serum pool of three p21-35-immunized mice was diluted 1:100 and added in duplicates to peptide-or *rDer p2*-coated plates. Binding is expressed as net absorbency after subtracting values obtained with the same dilution of normal mouse serum. The experiments were repeated twice with similar results.

2.4 p21-35 B and T cell epitopes partially overlap

The presence of both a T and B cell epitope on p21-35 prompted us to determine to what extent the two epitopes overlapped. To map precisely the two epitopes within p21-35, we made use of overlapping, 12-amino

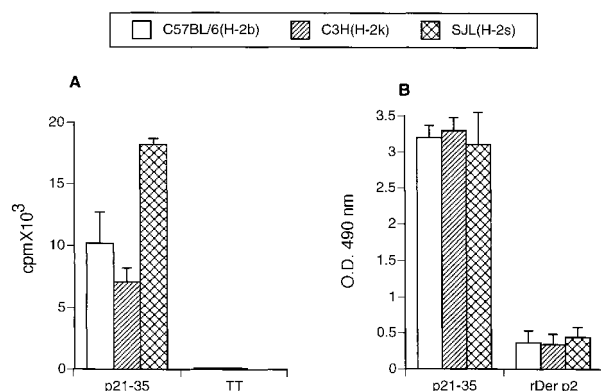


Fig. 3. T and B cell responses to p21-35 in three mouse strains with different MHC haplotypes. Six to eight mice from each strain (H-2^b, H-2^k and H-2^s) were immunized with p21-35. (A) T cells were pooled in each group and tested for *in vitro* reactivity against p21-35 (30 µg/ml). TT was used as negative control. Results are expressed as mean cpm (+ SD) of triplicates after subtracting mean cpm obtained from wells without peptide. (B) A 1/100 serum dilution was tested in duplicates for recognition of p21-35 and *rDer p2*. Binding is expressed as net absorbency after subtracting values obtained with the same dilution of normal mouse serum. Values are expressed as mean OD (+ SD) of five mice from the same groups.

acid-long peptides encompassing the whole 21-35 sequence with one amino acid offset from the previous peptide. Soluble peptides were used for T cell proliferation assays and biotin-labeled peptides were used to delineate the B cell epitope by ELISA after immobilization on neutravidin-coated plates. Fig. 4A shows the results of the T cell proliferation assay carried out with regional LN T cells from p21-35-immunized BALB/c mice. A significant proliferative response was observed with peptides 20-31 to 24-35 (30 µg/ml), indicating that the amino acid sequence 24-31(SEPCIIHR) was essential for the constitution of the T cell epitope. Fig. 4B shows that Ab of mice immunized with p21-35 recognized peptides 20-31 to 28-39, indicating that amino

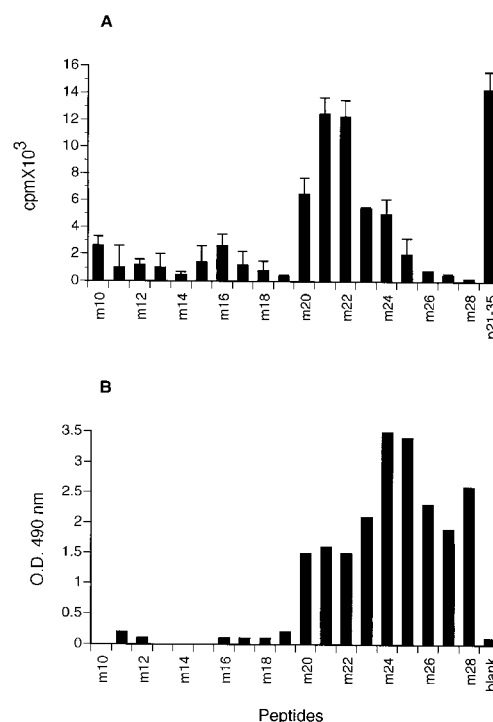


Fig. 4. T cell and B cell epitope mapping on p21-35. (A) T cells were obtained and pooled from five p21-35-immunized mice and tested for *in vitro* reactivity against a panel of synthetic 12-mer peptides (30 µg/ml) covering the *Der p2* sequence residues 10 to 39, with one amino acid offset from the previous peptide. Numbers given on the horizontal axis correspond to the N-terminal peptide residue. Results are expressed as mean cpm (+ SD) of triplicates after subtracting the mean cpm obtained from wells without peptide. (B) Anti-p21-35 pooled mouse sera were tested in duplicates against a panel of synthetic peptides encompassing residues 10 to 39 with one amino acid offset from the previous peptide. Binding is expressed as net absorbency after subtracting values obtained with the same dilution of normal mouse serum. The experiments were carried out in duplicates and repeated two times with similar results.

acid sequence 28–31 (IIHR) was sufficient for the binding of Ab. A mouse mAb (C10H7) produced towards p21–35 exhibited similar binding characteristics, *i.e.* it recognized amino acid sequence 28–35 (data not shown).

2.5 B and T cell epitopes exhibit distinct critical amino acid residues

As B and T cell epitopes partially overlap, we went on determining whether they shared critical amino acid residues. For the T cell epitope, we first identified the putative binding motifs for Ia molecules (H-2^d) using known algorithms [19]. Glu25, Ile28, His30 and Lys33 were

selected as such for positions 1 (P1), 4 (P4) 6 (P6) and 9 (P9), respectively. Since the residue at P1 appears to be degenerated, we selected Ile28 as a target residue for substitution and produced two mutants with substitution Ile28Asn and Ile28Ala/Phe35Ala, respectively. Two other mutants were used as controls, carrying substitutions located outside of the main T cell epitope core, namely Gly23 and Gly32, each replaced with a Thr (Fig. 5 A). Proliferation assays showed that substitutions of Ile28 and Ile28+Phe35 completely abrogated the response obtained with p21–35. Substitution at positions 23 and 32 somewhat reduced but did not abolish the proliferative response (Fig. 5 B).

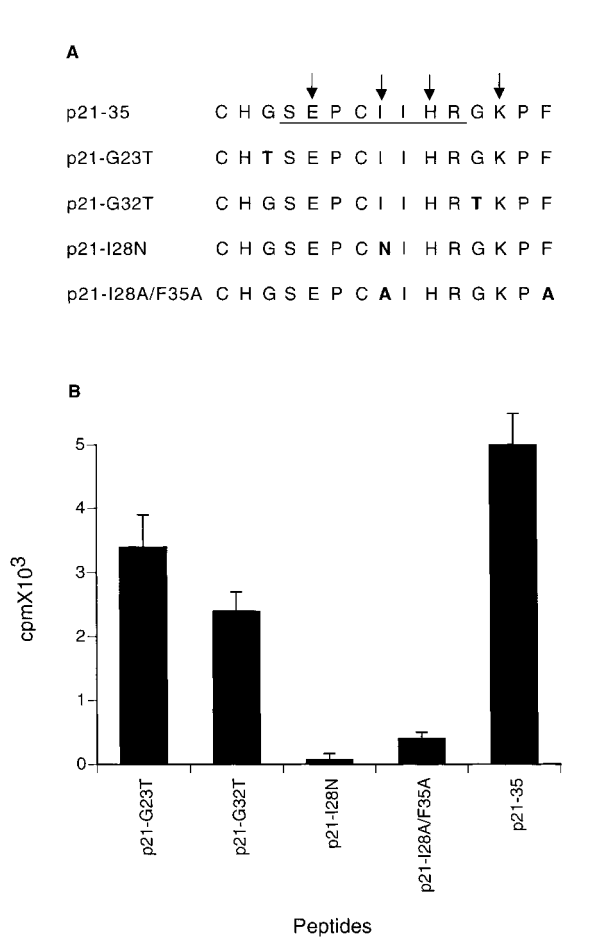


Fig. 5. T cell proliferative response to p21–35. (A) Sequence of wild-type and the four mutants of p21–35. The core epitope sequence is underlined with arrows indicating residues that were predicted to bind MHC class II molecules (H-2^d). (B) A T cell pool from five p21–35-immunized mice was tested for reactivity towards p21–35 or each of the four mutants (30 µg/ml). Results are expressed as mean of triplicates (+ SD) after subtracting mean cpm obtained from control wells without peptide.

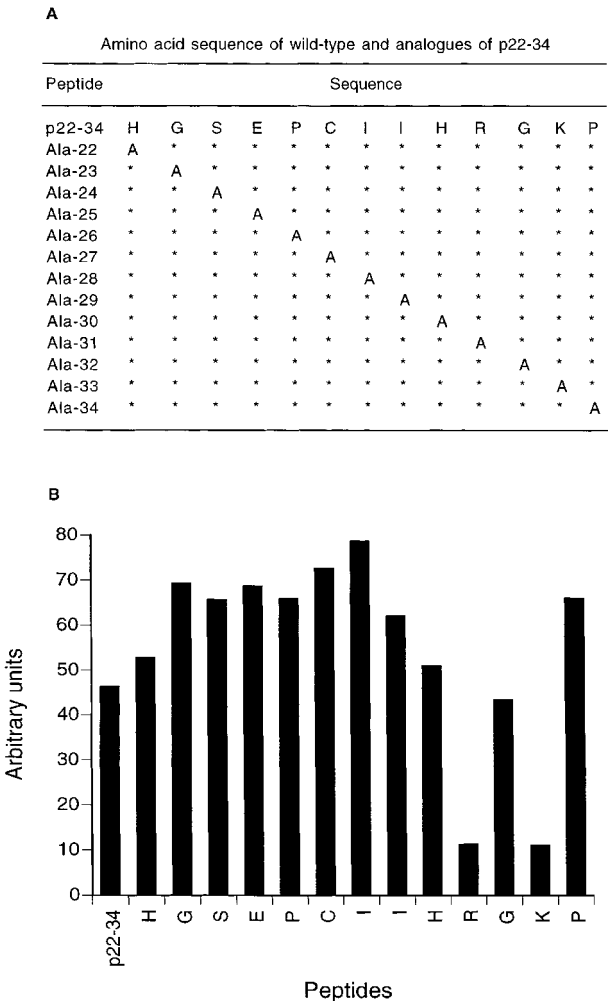


Fig. 6. Binding of mAb C10H7 to p22–34 or analogue peptides. (A) Amino acid sequence of wild-type and analogues of p22–34. (B) Binding of mAb C10H7 (5 µg/ml) to p22–34 and analogue peptides. Unit values were computerized after densitometric scanning of the membrane. Letters shown on the horizontal axis correspond to substituted positions.

An Ala-scan strategy was used to determine the critical residues for Ab binding. Since a conservative estimate of B cell epitope encompassed residues 28–35, mAb C10H7 was used for further mapping. Thirteen-mer peptides were constructed on a cellulose membrane in which each amino acid of the 22–34 peptide was substituted in turn by Ala (Fig. 6 A). The results given in Fig. 6 B show that two residues, Arg31 and Lys33, were crucial, as Ab binding to Arg31Ala or to Lys33Ala was reduced by fivefold. Interestingly, it can be seen that substitution by Ala of some amino acids such as Ile28 resulted in an increased mAb binding.

2.6 The B cell response can be abrogated

Based on the above experiments, it appeared possible to produce analogues of p21–35 which would activate T cells but not B cells, thereby fulfilling the requirements for a safe candidate for T cell anergy-based immunotherapy. Thus, three mutants of p21–35 were synthesized, in which residues Arg31, Lys33 or Arg31/Lys33 were substituted by Ala, respectively. BALB/c mice were immunized in the footpad with such mutant peptides. The results indicate that mice immunized with the p21-Arg31Ala mutant maintain their capacity to produce Ab towards p21–35 (Fig. 7 B), while exhibiting a much reduced capacity to recognize the T cell epitope of wild-type p21–35 (Fig. 7 A), suggesting the recruitment of an alternative T cell epitope. By contrast, mice immunized with mutant peptide p21-Lys33Ala still showed T cell proliferation in response to the wild-type peptide and to *rDer p2* (Fig. 7 A), but have lost the capacity to elicit specific Ab to the same antigen (Fig. 7 B). The mutant peptide p21-Arg31Ala/Lys33Ala lost their capacity to activate T cells and B cells (Fig. 7, A and B).

3 Discussion

Immunotherapeutic strategies based on the use of T cell epitope-containing peptides to treat allergic or autoimmune diseases have recently become popular. Such peptides, which can readily be selected by T cell proliferation assays, have the capacity to induce T cell anergy in a highly specific manner if they are presented to T cells in the absence of co-stimulatory signals. Induction of T cell anergy has been attempted in a number of studies, including clinical, with variable but promising results [6, 7, 10]. However, in some cases such peptides were rather unexpectedly shown to induce the production of specific Ab [16].

We have investigated the immunogenicity of a 15-mer peptide containing a major T cell epitope of *Der p2*, one

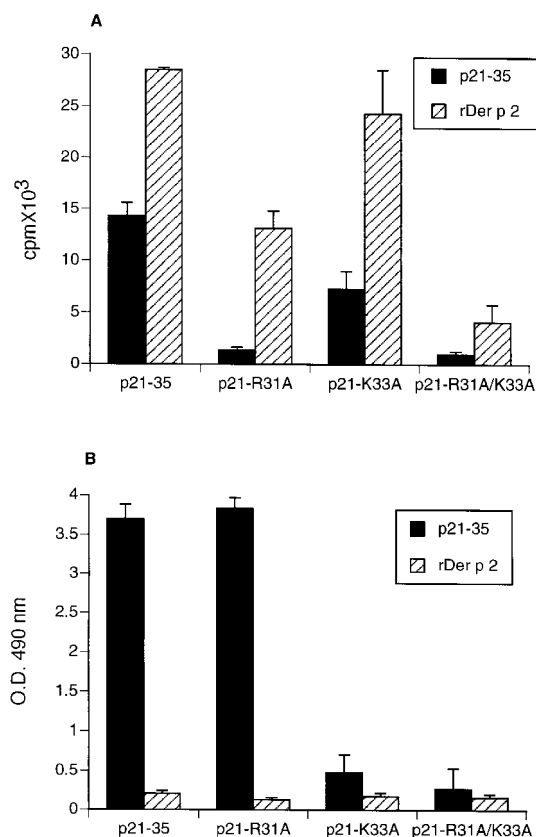


Fig. 7. Comparison of T cell and B cell reactivities to p21–35 and *rDer p2* using three mutant peptides. Groups of five mice were immunized with p21–35, p21-Arg31Ala, p21-Lys33Ala or p21-Arg31Ala/Lys33Ala, respectively. (A) T cells were pooled in each group and tested for *in vitro* reactivity against p21–35 and *rDer p2* (30 µg/ml each). Results are expressed as mean (+SD) of triplicates after subtracting mean cpm obtained from wells without peptide. (B) A 1/100 serum dilution was tested in duplicates for recognition of p21–35 and *rDer p2*. Binding is expressed as net absorbency after subtracting values obtained with the same dilution of normal mouse serum. Values are expressed as mean OD (+SD) of five mice from the same group.

of the main allergens of the house dust mite *D. pteronyssinus*, and show that such peptide contains both a T and B cell epitope. Similar results were obtained in the context of four different mouse MHC haplotypes, suggesting that p21–35 could behave as a universal T cell epitope. Preliminary experiments made with human T cells have indeed confirmed this prediction (our unpublished data). The B and T cell epitopes partially overlap while keeping full function. Critical residues for T cell activation (and/or MHC class II anchorage) and Ab recognition were nevertheless distinct, allowing us to construct mutants with isolated B or T binding properties.

Short peptides can elicit Ab production only when carrying three recognition sites, *i. e.* sites recognized by B cell surface Ig, MHC class II molecules and T cell $\alpha\beta$ receptors, which is apparently the case for p21–35, as judged from its immunogenic properties.

From the results obtained using overlapping peptides it was possible to identify the sequence SEPCIIHR as the core epitope for T cell recognition. Residues critical for H-2^d MHC class II anchoring were then identified using available algorithms [19]. Assuming that residue Glu25 corresponds to the relative P1 position, residues involved in MHC binding would be found at P4 and P6, namely Ile28 and His30, respectively [19]. These amino acids can be direct contact moieties, but may also indirectly influence peptide binding by altering the conformation of adjacent amino acid side chains [17, 20]. The amino acid at P9 (Lys33) is another predicted anchoring residue, which is located outside of the core T cell epitope of p21–35, but still within the MHC II peptide binding groove. P4 and P9 generally contribute to peptide binding, while other positions such as P6 further restrict the specificity of binding [21]. The degeneracy of P1 anchoring positions for I-A molecules might explain the apparently short (six to seven residues) minimal core sequence of peptides binding to I-A molecules [22]. Our data suggest the importance of Ile28 for p21–35 binding to I-A^d molecules, which interestingly, is similar to findings in atopic subjects [23]. The presence of Lys at position 33 is, however, less critical as the p21-Lys33Ala analog peptide maintains T cell activation properties. Moreover, the fact that Gly23Thr and Gly32Thr mutants of p21–35 significantly decreased T cell proliferative response demonstrates that residues outside the motif sequence may also substantially affect peptide binding to MHC II molecules, and as such exert an influence on the T cell response [24].

The results of the Ala scanning study showed that amino acids Arg at position 31 and Lys at position 33 are required for maintaining the B cell epitope within p21–35, since Ab binding was very much reduced when Arg31 or Lys33 was substituted by Ala. On the other hand, the magnitude of mAb C10H7 binding to some of the mutant peptides (Ala23, Ala25, Ala27 and Ala28) was higher than binding to the corresponding wild-type peptide under the same assay conditions. In particular, a 70 % higher response was recorded in the Ala28 peptide. Our work provides evidence that in the complex of the mAb C10H7 with peptides, the key binding contacts are provided by Arg31 and Lys33, but that the affinity of binding is most readily improved by altering other residues. Similar results have been reported [25]. Two categories of amino acids can therefore be identified in p21–35: (1) amino acids that determine Ab affinity and

specificity, such as Arg31 and Lys33, and; (2) amino acids that can be accommodated by Ab but contribute to decrease the binding affinity, such as Ile28.

We show in the present study that a short peptide that contains partially overlapping T and B cell epitopes has the capacity to trigger both T cell proliferation and Ab production. Our data show that the T cell epitope shares four amino acids (28, 29, 30 and 31) with the B cell epitope within p21–35, pointing to the amino acid sequence IIHR as pivotal for both T cell proliferation and Ab binding. Interestingly, Ile28 was identified as a key amino acid for T and B cell epitope and possibly MHC II anchoring, as substitution of Ile28 eliminated the capacity of inducing T cell proliferation, while increasing the binding capacity for Ab. It is commonly admitted that optimal responses are obtained when T and B cell epitopes are located at some distance from each other [26]. By contrast, complete overlap between the two epitopes seems to abrogate Ab formation [11], although a number of exceptions have been reported [12]. Partial overlap between a B and a T cell epitope poses an interesting case in point, in particular when the same amino acid substitutions abrogate both T and B cell activation capacities. Such partial overlap is thought to impair the production of Ab [27] but again exceptions have been reported [12, 28]. We offer here another example of an efficient immune response with a peptide containing partially overlapping B and T epitopes. The reason why an overlap of epitopes decreases the efficiency of an immune response is likely related to the fact that binding and internalization of a peptide through B cell-specific surface Ig block the transfer of the peptide to MHC class II molecules [26]. However, as the initial T cell priming depends on antigen presentation by dendritic rather than B cells, such priming is independent of surface IgG recognition. Primed T cells can then provide specific B cells with appropriate help towards Ab production.

We conclude that T cell epitope-containing peptides contemplated for use in immunotherapy of allergic or autoimmune diseases may also contain B cell epitopes. Injection of peptides having characteristics similar to that of p21–35 can elicit the production of specific Ab which, in presence of the right cytokine environment [29], could include specific IgE Ab and, hence, a risk of anaphylaxis. Any T cell epitope-containing peptide, in particular whenever used in combination, should be checked for the presence of B cell binding sites.

Our data show that it is possible, at least in some cases, to derive mutant peptides with only T cell-activating and/or -nergizing properties, even in situations in which B and T cell epitopes overlap. Such results provide potentially important information for the design and use of peptide vaccines.

4 Materials and methods

4.1 Animals

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, Netherland. Female mice 6–8 weeks of age were used in all studies.

4.2 Synthetic peptides

Three 15-mer peptides covering *Der p2* amino acids 11–45 with five overlapping amino acids and a panel of peptides (m10–m39) containing 12 amino acids covering *Der p2* amino acids 10 to 39 with one amino acid offset from previous peptide are listed in Table 1. For the use of NeutravidinTM-coated ELISA plates (see below), peptides m10–m39 were labeled with biotin. All synthetic peptides were purified and labeled by Eurogentec (Seraing, Belgium) based on the known *Der p2* sequence [30]. The purity was $\geq 85\%$.

4.3 Production of *rDer p2*

Der p2 mRNA obtained from mite culture extract was transcribed into cDNA and amplified by PCR using specific *Der p2* primers. The products of amplification were

cloned into the pGE4T-2 vector and introduced into the *Escherichia coli* strain DH5 α . Positive colonies were chosen and amplified. Plasmid DNA was prepared by PCR with primers specific for *Der p2*. Expression of the *rDer p2* protein was induced by the addition of isopropyl- β -thiogalactopyrenoside (IPTG) and produced by growth of positive colonies. The glutathione S-transferase-fused *Der p2* was identified by sequencing and molecular weight determination and reactivity with a panel of specific Ab produced in our laboratory.

4.4 ELISA

4.4.1 Assay for polyclonal Ab in mouse serum and mAb in culture supernatant

Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with peptide diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6, by an overnight incubation at 4 °C, and blocked with 1 % skimmed milk in PBS-Tween-80 (PBST). After washing, serum (1:100) or hybridoma supernatant was added and incubated for 1 h at 37 °C. The plates were washed again before incubation with peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, CA) diluted 1:2000 for 1 h at 37 °C followed by the substrate (o-phenylenediamine). The reaction was stopped by addition of 2 N H₂SO₄. Absorbency was read at 490 nm and 650 nm with a Precision Microplate Reader. Coefficients of variation (CV) of < 10 % were obtained.

4.4.2 NeutravidinTM-coated ELISA plate

Plates (Pierce, Rockford, IL) were incubated with 1 μ g/ml biotin-labeled peptide for 2 h at room temperature in 100 μ l PBS, pH 7.2. The plates were washed in PBST and blocked with 0.5 % PBS-BSA. After washing, serum (1:100) was added and incubated for 2 h at room temperature. The plates were washed and the binding of Ab was evaluated as above using peroxidase-conjugated goat anti-mouse IgG. Such an assay procedure gives CV of < 5 %.

4.5 Ala-scan

p22–34 and a set of 13-mer analogue peptides substituted by Ala were synthesized on a cellulose membrane by the spot method [31]. The membrane was washed with TBS buffer, and then incubated overnight with blocking buffer. After washing with TBS-Tween-20 (TBS-T), the membrane was incubated with Ab diluted in blocking buffer for 90 min at 37 °C. Alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad) diluted at 1:2000 was then added and incubated for 90 min. After washing with TBS-T followed by Tris pH 8.5, the substrate was added to the membrane for 30 min, color intensity was recorded by scanning and quantitated by image analysis.

Table 1. Sequences of peptides

Peptide	Position	Sequence
p11	11–25	HEIKKVLVPGCHGSE
p21	21–35	CHGSEPCIIHRGKPF
p31	31–45	RGKPFQLEAVFEANQ
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	PGCHGSEPCIIH
m20	20–31	GCHGSEPCIIHR
m21	21–32	CHGSEPCIIHRG
m22	22–33	HGSEPCIIHRGK
m23	23–34	GSEPCIIHRGKP
m24	24–35	SEPCIIHRGKPF
m25	25–36	EPCIIHRGKPFQ
m26	26–37	PCIIHRGKPFQL
m27	27–38	CIIHRGKPFQLE
m28	28–39	IIHRGKPFQLEA

4.6 T cell purification from draining LN

Mice were immunized s.c. in the footpads with 50 µg peptide emulsified in CFA and boosted at day 14 with the same dose of antigen in IFA. Ten days later, popliteal LN were removed, a single-cell suspensions were obtained. T cell-enriched fractions of these LN cells were prepared by negative selection using the MACS[®] system and anti-CD45R, anti-CD11b and anti-CD11c Ab (all conjugated to magnetic beads), according to the manufacturer's specifications (Miltenyi Biotec GmbH, Germany). The purity of the T cell suspension was usually $\geq 97\%$.

4.7 T cell proliferation assay

Purified T cell suspensions were adjusted at a concentration of 4×10^6 cells per ml in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U penicillin per ml, 100 µg streptomycin per ml, 5×10^{-5} M 2-ME, and 10% heat-inactivated FCS. Of the T cell-enriched fraction, 100 µl (4×10^5 cells per well) was cultured in 100 µl of the medium-described above containing different concentrations of test peptide and irradiated syngeneic naive spleen cells as a source of APC (4×10^5 per well). Incubation was carried out for 4 days in a humidified atmosphere of 5% CO₂ in air. Cultures were pulsed during the final 18 h incubation with 1 µCi [³H] thymidine per well. Cells were then harvested onto glass fibers by using a multiple-cell harvester. Radioactivity incorporated into proliferating cells was determined in a liquid scintillation counter. All tests were carried out in triplicates. The proliferative response was expressed as $\Delta \text{cpm} \times 10^{-3}$ after subtracting background value of wells without antigen.

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