

Discontinuous IgE-binding epitopes contain multiple continuous epitope regions: results of an epitope mapping on recombinant Hol 1 5, a major allergen from velvet grass pollen

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Summary

Background The knowledge of IgE-binding epitopes on allergen molecules is important for better understanding allergen–antibody interactions and, thus, for developing new strategies for immunotherapy.

Objective Our purpose was to more precisely define the number and structure of IgE-binding epitopes of a paradigmatic major grass pollen allergen.

Methods We performed an IgE-binding epitope mapping of rHol 1 5, a group V pollen allergen of velvet grass (*Holcus lanatus*), with overlapping fragments (length between 15 and 186 amino acids), which were expressed in *E. coli* as MBP fusion proteins. Using sera of 65 grass pollen allergic patients, the fragments were analysed by immunoblotting for IgE reactivity. Specificity of antibody binding was confirmed by competitive blot inhibition assays.

Results At least four different continuous IgE-binding epitopes were identified on small fragments (about 30 amino acids), and at least five different discontinuous IgE-binding epitopes on larger fragments, which were destroyed by further fragmentation. The fragments were differentially recognized by individual patients' sera. By investigating IgE-binding to one of the small fragments in more detail, we found further epitope regions on this fragment. It was noteworthy that IgE reactivity to small fragments was weak compared to large fragments or to the complete molecule. Competitive blot inhibition experiments showed that binding of IgE antibodies to the small fragments was specific but with lower avidity than to the complete rHol 1 5.

Conclusion rHol 1 5 harbours multiple discontinuous as well as continuous IgE-binding epitopes spread over the whole molecule, which were individually recognized by IgE antibodies from different patients. Low avidity of IgE antibodies to small fragments suggests that the continuous epitope regions do not represent the complete epitope and are most probably parts of discontinuous epitopes.

Keywords: grass pollen allergen, velvet grass, IgE-binding epitopes, epitope mapping, continuous/ discontinuous epitopes

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Introduction

Type I allergy which affects up to 20% of the population of industrialized countries is often induced by airborne allergens derived from grass and tree pollen, house dust mite and animal dander. Allergic patients react disproportionately to these otherwise harmless environmental molecules by production of IgE antibodies. Investigation of the IgE-binding epitopes on allergens aims at a better understanding of allergen-antibody interaction and may contribute to the development of more effective strategies for immunotherapy. Several strategies have been proposed: (i) the development of modulatory or blocking antibodies [1]; (ii) the application of IgE-haptens, that means peptides able to bind monovalently to IgE, thus inhibiting the cross-linking of IgE receptors [2]; and (iii) the use of allergen variants lacking IgE-binding epitopes, thus, preventing negative side-effects during immunotherapy [3,4].

B cell epitopes are divided into two classes: (i) continuous epitopes, where short linear stretches of residues are recognized; and (ii) discontinuous epitopes, where sets of residues are brought together by folding of the protein [5]. Due to the tertiary structure of proteins most of the B cell epitopes are supposed to be discontinuous [5,6]. The exact structure of such epitopes can only be elucidated by X-ray crystallography of antigen-antibody complexes [7], but until now only a few known allergens have been crystallized [8–10] and none of them in conjunction with an antibody. Another approach is to analyse the binding of antibodies to allergen fragments or allergen mutants, but one has to bear in mind that fragmentation or mutation can destroy discontinuous epitopes. This effect has been shown for the birch pollen allergen Bet v 1 and the house dust mite allergens Der p 1 and Der p 2, which possess mainly discontinuous IgE-binding epitopes, that were destroyed by fragmentation or mutagenesis of cysteine residues responsible for the formation of disulphide bonds [3,11,12].

Despite these limitations, IgE-binding epitopes could be identified on peptides of food allergens such as the peanut allergens Ara h 1, Ara h 2 and Ara h 3 [13–15], and the cod fish allergen M [16], and also on fragments of grass pollen allergens from different grass species [17–20]. At least two different IgE-binding epitopes were shown on the group V pollen allergens of timothy grass and rye grass, Phl p 5a and Lol p 5a and b, respectively. Group V grass pollen allergens display very high homology between each other. To systematically analyse the presence of IgE-binding epitopes on a representative group V allergen, we therefore performed an extensive mapping on the recombinant group V pollen allergen of velvet grass, rHol 1 5. Using 65 patients' sera, multiple discontinuous as well as continuous IgE-binding epitopes were identified, which are spread over the whole molecule and were individually recognized by the sera.

Materials and methods

Pollen extracts and patients' sera

Extract of velvet grass pollen was prepared as described elsewhere [21]. Sera from 65 grass pollen allergic patients were selected according to their IgE reactivity to group V allergens in extracts from velvet grass pollen (CAPscore 4–6). The sera were collected in the Medical Clinic Borstel, the University Hospital Bergmannsheil, Bochum, the Central Institute of Transfusion Medicine Eilbek and the Institute of Immunology and Transfusion Medicine, Medical University, Lübeck. All sera reacting to natural Hol 1 5 in velvet grass pollen extracts recognized also the recombinant Hol 1 5.01 isoform.

PCR cloning of rHol 1 5.01 fragments

As a template for PCR cloning of rHol 1 5.01 fragments we used the previously cloned cDNA coding for the group V allergen from velvet grass pollen rHol 1 5.01 (accession number Z97874) [22]. For PCR gene-specific primers were designed with an AT-clamp and restriction sites (Table 1) for cloning into the pMAL-c2 expression vector (New England Biolabs, Beverly, MA). Restriction enzyme *Bam*HI was used for the coding strand, and *Hind*III for the complementary strand (MBI Fermentas AB, Vilnius, Lithuania). Primers were purchased from Biosource, Fleurus, Belgium. PCR was performed on the thermocycler from Perkin Elmer (Norwalk, Connecticut) under the following conditions: 1 ng DNA template, 1 µM primers, 200 µM dNTPs, 1 U *Taq*Plus Long Polymerase (Stratagene, LaJolla, CA), and buffer #7 from Opti-Prime PCR Optimization Kit (Stratagene, LaJolla, CA) plus 1% DMSO for 30 cycles: denaturation 1 min at 96 °C, annealing 30 s at 58 °C and extension 1 min at 72 °C. PCR products were digested with enzymes *Bam*HI and *Hind*III and ligated into the pMAL-c2 expression vector forming fusion proteins with the maltose-binding protein (MBP). *E. coli* JM 109 cells were transduced by respective plasmids. Transformants were checked by plasmid preparation followed by restriction analysis and double-stranded sequencing on ABI 377 using the universal and the male primer (New England Biolabs, Beverly, MA).

Small overlapping peptides (N-Peps) covering the N-terminal fragment F16 were cloned by annealing of the appropriate 64 bp-oligonucleotides (Biosource, Fleurus, Belgium) (Table 2) under the following conditions: 3 µg of sense and antisense oligonucleotide pairs were denatured at 95 °C for 5 min and annealed by slowly cooling to room temperature for 15 min. Double-stranded fragments were digested and ligated into the pMAL-c2 expression vector using the *Bam*HI/*Hind*III restriction sites as described above.

Table 1. Sequences of primers used for cloning of rHol I 5.01 fragments in pMAL-c2

Primer	Sequence [5' → 3']	Position [bp]
P 1s	ATAT <u>GGA TCC ATC GAG GGT AGG</u> GCA GAC GCC GGA TAT ACA C	1–19
P 1a	ATAT <u>AAG CTT</u> ATCAGCAAATCACTCAAC	884–866
P 4.2	ATAT <u>GGA TCC ATC GAG GGT AGG</u> TTC GTT ACC ACC CTC ACC GAG GCG	304–327
P IV	ATAT <u>GGA TCC ATC GAG GGT AGG</u> AAG TTC ACC GTC TTC GAG G	490–508
P III	ATAT <u>GGA TCC ATC GAG GGT AGG</u> GTC AAG CAG GCC TAC GCC G	589–607
P II	ATAT <u>GGA TCC ATC GAG GGT AGG</u> AAG GAG GCC AAG CCT GTT	694–711
P 431S	ATAT <u>GGA TCC ATC GAG GGT AGG</u> AAC GCC GGC TTC AAG ACG	89–108
P gsp2	ATAT <u>AAG CTT</u> TCA AAA GAC GGT GTA CTT GAC CT	648–629
P F4s	ATAT <u>GGA TCC ATC GAG GGT AGG</u> GAG GCG CTC CGC GTC AT	322–338
P 4.1	ATAT <u>GGA CTT ATC GAG GGT AGG</u> GAG GCC ACC GAG GAG GTC GGC GCG	373–393
P P2	ATAT <u>AAG CTT</u> TCA GTT GAC GGC GGT CTC GAG G	154–576
P F4a	ATAT <u>AAG CTT</u> TCA GAC GGT GAA CTT GTC GTT G	501–483
P P5s	ATAT <u>GGA TCC ATC GAG GGA AGG</u> GCC AAC GTC CCT CCG GCG	124–141
P P5a	ATAT <u>AAG CTT</u> TCA GAC GGC GTG GAC CTC GAG GG	360–347
P NT2	ATAT <u>AAG CTT</u> TCA GGC GGC CTC GAA GGT CTT GTA	168–148
P 2s	ATAT <u>GGA TCC ATC GAG GGT AGG</u> CAG AAG CTG CTC GAA GAC G	70–88
P P1	ATAT <u>AAG CTT</u> TCA GTC TTC GAG CAG CTT CTG	87–70

Restriction sites are underlined, stop codons are italic faced and cleavage sites for factor Xa are bold faced. Position refers to the nucleotides of the cDNA clone rHol I 5.01 (accession number Z97874).

Expression of recombinant fragments as MBP fusion proteins

Recombinant fragments of rHol I 5.01 were expressed as MBP fusion proteins. Expression was induced in *E. coli* cell cultures grown to an optical density of $A_{600} = 0.8$ by

0.3 M isopropyl thiogalactoside (IPTG). After incubation for additional 2 h at 37 °C, cells were harvested by centrifugation. The cell pellet of 1 mL of culture was resuspended in 100 µL of reducing SDS sample buffer and immediately subjected to SDS-polyacrylamide gel electrophoresis (PAGE) or stored at –20 °C until use.

Table 2. Sequences of oligonucleotides used for cloning of small peptides (N-Peps) of the rHol I 5.01-fragment F16

Oligonucleotide	Sequence [5' → 3']	Position [bp]
N-Pep 1s	ATAT <u>GGA TCC</u> CAG AAG CTG CTC GAA GAC GTC AAC GCC GGC TTC AAG ACG GCC GTG TGA <u>AAG CTT</u> ATAT	70–114
N-Pep 1a	ATAT <u>AAG CTT</u> TCA CAC GGC CGT CTT GAA GCC GGC GTT GAC GTC TTC GAG CAG CTT CTG <u>GGA TCC</u> ATAT	114–70
N-Pep 2s	ATAT <u>GGA TCC</u> GTC AAC GCC GGC TTC AAG ACG GCC GTG GCC GCC GCG GCC AAC GTC TGA <u>AAG CTT</u> ATAT	88–132
N-Pep 2a	ATAT <u>AAG CTT</u> TCA GAC GTT GGC CGC GGC GGC CAC GGC CGT CTT GAA GCC GGC GTT GAC <u>GGA TCC</u> ATAT	132–88
N-Pep 3s	ATAT <u>GGA TCC</u> ACG GCC GTG GCC GCC GCG GCC AAC GTC CCT CCG <u>GCG GAC</u> AAG TAC TGA <u>AAG CTT</u> ATAT	106–150
N-Pep 3a	ATAT <u>AAG CTT</u> TCA GTA CTT GTC CGC CGG AGG GAC GTT GGC CGC GGC GGC CAC GGC CGT <u>GGA TCC</u> ATAT	150–106
N-Pep 4s	ATAT <u>GGA TCC</u> GGC AAC GTC CCT CCG GCC GAC AAG TAC AAG ACC TTC GAG GCC TGA <u>AAG CTT</u> ATAT	124–168
N-Pep 4a	ATAT <u>AAG CTT</u> TCA GGC GGC CTC GAA GGT CTT GTA CTT GTC CGC CGG AGG GAC GTT GCC <u>GCG TCC</u> ATAT	168–124

Restriction sites are underlined, and stop codons are italic faced. Position refers to the nucleotides of the cDNA clone rHol I 5.01 (accession number Z97874).

Immunoblot analysis

One $\mu\text{L}/\text{cm}$ bacterial lysate was separated on 12% SDS-PAGE according to Laemmli [23]. Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by semidry blotting for 30 min at $0.8 \text{ mA}/\text{cm}^2$ [24] and membranes were incubated in 0.1 M Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 for blocking of free binding sites. For protein staining the membrane was incubated with 0.1% India Ink (Pelikan AG, Hannover, Germany) [25]. Immunological staining was performed with patients' sera diluted 1 : 20 in Tris-Tween. Alkaline phosphatase-conjugated monoclonal antihuman IgE (1 : 2000) (Allergopharma KG, Reinbek, Germany) was used as secondary antibody.

B cell epitope prediction

Potential antigenic determinants were predicted by two different methods: (i) the hydrophilicity profile according to Hopp and Woods [26] (PCGene, Intelligenetics, Geel, Belgium); and (ii) the antigenic index according to Jameson and Wolf [27].

Blot inhibition assays

For blot inhibition assays fusion proteins MBP-Hol 1 5.01, MBP-F5, MBP-F10, MBP-F14 and MBP-F16 and MBP alone as negative control were purified by amylose resin according to the manufacturers' instructions (New England Biolabs, Beverly, USA). Velvet grass pollen extract (g13) ($5 \mu\text{g}/\text{cm}$) and recombinant fusion proteins (MBP-Hol 1 5.01: $1 \mu\text{g}/\text{cm}$, MBP-fragments: $10 \mu\text{g}/\text{cm}$) were separated by SDS-PAGE and blotted onto nitrocellulose membrane as described above. Patients' sera were pre-incubated for 1 h with the respective inhibitor at different concentrations (g13: 2, 4, 8, or 12 mg/mL serum; MBP-Hol 1 5.01: 20, 40, 80, or 800 $\mu\text{g}/\text{mL}$ serum) and then incubated with the nitrocellulose strips. For comparison of the IgE binding to the complete allergen and the fragment F16 the fusion proteins MBP-F16 ($10 \mu\text{g}/\text{cm}$) or a mixture of MBP-F16 ($10 \mu\text{g}/\text{cm}$) plus MBP-Hol 1 5.01 ($1 \mu\text{g}/\text{cm}$) were separated by SDS-PAGE and blotted onto nitrocellulose as described above. Nitrocellulose strips of MBP-F16 and a mixture of MBP-F16 + MBP-Hol 1 5.01 were incubated with patients' sera reactive to F16 and immunological detection was done as described above.

Results

Cloning and expression of recombinant fragments

By PCR we derived 17 overlapping fragments of the Hol 1

5-isoform rHol 1 5.01 covering the whole allergen molecule, schematically shown in Fig. 1. To get efficient comparable expression rates also of small peptides of 15 amino acids length we expressed the complete rHol 1 5.01 and the fragments as fusion protein with the *E. coli* maltose-binding protein (MBP). Expression rates were controlled by Coomassie Blue staining of proteins after separation by SDS-PAGE as well as by protein staining with India Ink and AuroDye of Western blots (data not shown).

Mapping of IgE-binding epitopes using recombinant PCR-derived fragments

After separation by SDS-PAGE bacterial lysates harbouring the differently sized fragments were blotted onto nitrocellulose membrane and analysed for IgE reactivity with sera of 65 allergic patients. Figure 1 shows the summary of the IgE reactivity in percentage of investigated sera. No IgE reactivity was observed to MBP alone or other components of the bacterial extracts (data not shown). Large fragments like F1, F2, F6, F7 and F8 spanning the C-terminus and the middle region of the molecule were recognized by all sera. The observation that the small, non-overlapping fragments shown in Fig. 1 (light grey bars) F5 (33 amino acids), F10 (35 amino acids), F14 (43 amino acids) and F16 (33 amino acids) were bound by patients' IgE indicates that each of them is carrying at least one continuous IgE-binding epitope. Continuous epitopes on the fragments F5, F10 and F14 can be designated as 'minor epitopes', because they were recognized by only 9, 19 and 24% of the sera, respectively. However, the N-terminal fragment F16 was recognized by nearly half of the sera (47%).

In addition to the four continuous epitopes, we detected at least five discontinuous IgE-binding epitopes on larger fragments by subtractive analysis (Fig. 1, broken lines). Concerning the C-terminus of the molecule, we observed that sera binding to fragment F5 are also reactive to fragments F4 and F3 because fragment F5 is part of these two larger fragments. However, fragment F4 was recognized by more patients than F5, namely 48%, indicating that at least one more epitope is present in this region. Furthermore, fragment F3 is recognized by 88% of the sera, some of them reacting also with fragment F4 and F5 and/or the overlapping fragment F9 and F10. There are also sera which do not recognize any of the smaller fragments covering region F3 indicating that there is another, possibly discontinuous epitope on this fragment, which is destroyed by cutting the fragment into smaller peptides.

Concerning the middle part of the molecule, we found that fragment F8 was bound by IgE of all patients. However, the two fragments F9 and F14, covering the region F8, were recognized only by 19 and 24%,

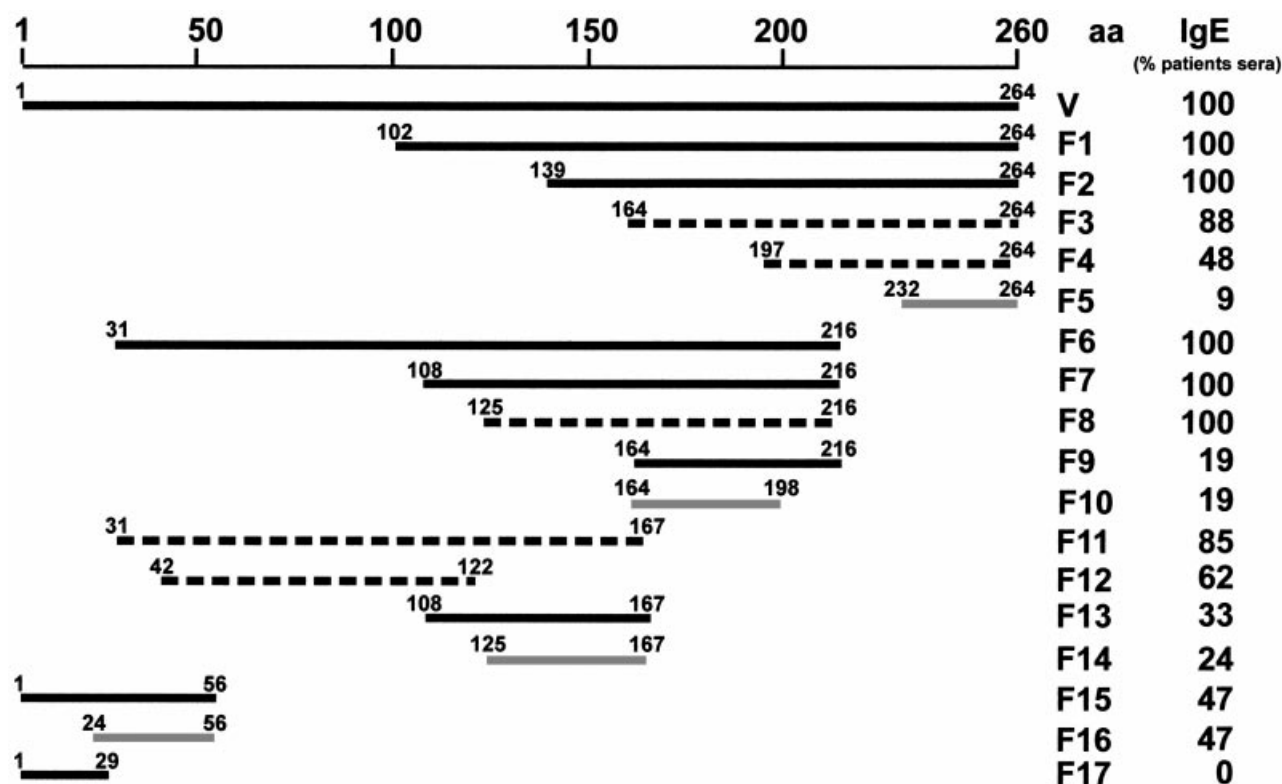


Fig. 1. Schematic map of the location of recombinant fragments on rHol I 5.01 (V). Small numbers indicate the first and last amino acid of the fragments. Fragments of special interest are highlighted by different grey tones or patterns. Small fragments harbouring potential continuous IgE-binding epitopes are light grey. Broken lines represent the smallest fragments harbouring at least one discontinuous IgE-binding epitope. The number of IgE-reactive sera is given in percentage of all investigated sera [$n = 65$].

respectively, indicating that the other sera recognize one more discontinuous epitope on F8. This suggests that fragment F8 harbours at least one discontinuous and two continuous IgE-binding epitopes. On the large fragments F11 (137 amino acids) and F12 (80 amino acids) at least two further discontinuous IgE-binding epitopes could be detected. Epitope mapping revealed patients' sera reactive to F11, but not to F12, F13 or F14, which are overlapping fragments covering F11. From these data it is concluded that F11 harbours at least two discontinuous (F11 only, F12) and one continuous (F14) IgE-binding epitopes. In summary, we detected at least four continuous and five discontinuous IgE-binding epitopes on the rHol I 5 molecule.

Individual IgE reactivity

As it is assumed by the presence of multiple different IgE-binding epitopes on rHol I 5.01, patients' sera did not show uniform IgE reactivity to the identified epitopes, but revealed a highly individual binding pattern. As an example, Fig. 2 shows the individual IgE reactivity of four different patients: IgE antibodies of one patient, PS-DB, bound to fragment F5 (f), while the others did not.

Fragments F9 (k) and F10 (l) were bound exclusively by IgE from PS-20. In contrast to the other sera, IgE from PS-638 did not bind to F12 (n), F15 (q) and F16 (r), but to F13 (o) and F14 (p). Interestingly, IgE reactivity was usually weaker to smaller fragments than to larger fragments or the complete molecule. An exception was F2 (c). Although this fragment was smaller than F1 (b), IgE binding was stronger and comparable to the binding to the complete rHol I 5.

Alteration of the IgE-binding pattern during several pollen seasons

Sera of six patients under study were analysed for changes in their IgE-binding patterns over a time course of 4 to 7 years. Interestingly, the individual binding pattern of the IgE antibodies of these patients remained constant over time. We did not observe additional specificities of the IgE antibodies during the course of the disease (data not shown).

Fine mapping of IgE-binding epitopes on the N-terminal fragment F16

To investigate whether there are further epitopes on one

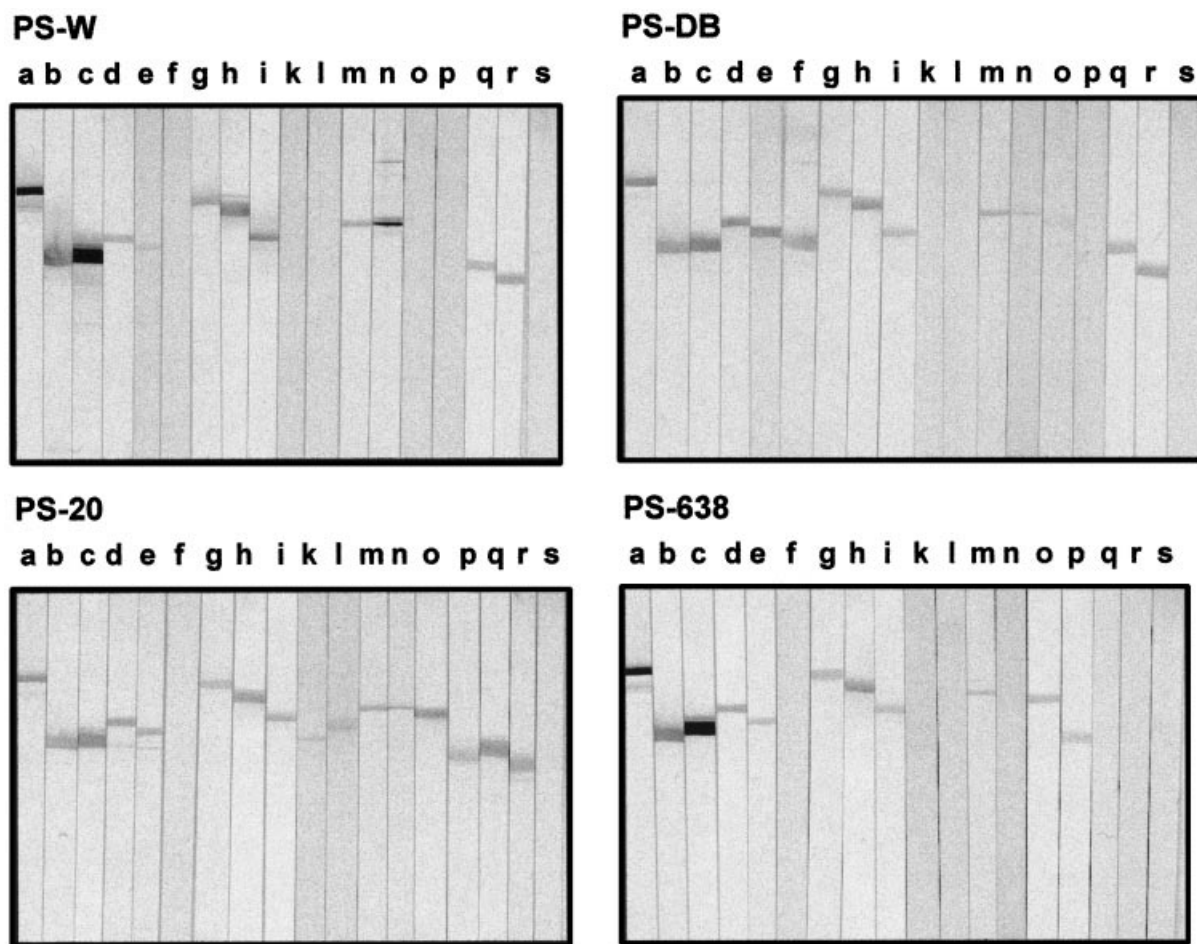


Fig. 2. Individual IgE-reactivity of four individual patients' sera (PS-W, PS-DB, PS-20, PS-638) with rHol I 5.01: (a) rHol I 5.01; (b – s) the fragments F1–F17.

small fragment we performed paradigmatically a fine mapping of IgE epitopes on the important N-terminal fragment F16, which was detected by 47% of the sera. Small overlapping peptides of 15 amino acids length with an increment of 6 covering the N-terminal fragment F16 were cloned and expressed in *E. coli* as MBP fusion proteins (N-Pep1–4). A schematic map of the fragments is shown in Fig. 3. Bacterial lysates were separated on SDS gels, blotted onto nitrocellulose membrane and analysed for IgE reactivity with 21 patients' sera reactive to the fragment F16. Interestingly, we observed six different IgE-binding patterns: (i) most of the sera (43%) did not react with any of the 15 amino acid peptides; (ii) 29% of the sera recognized N-Pep4; (iii) 14% of the sera bound to the peptides N-Pep1 and N-Pep2; (iv) 9% of the sera bound to all four peptides; (v) 5% of the sera showed binding only to N-Pep1; (vi) 5% bound to the peptides N-Pep1 and N-Pep4. The observation of six different IgE-binding patterns suggests the presence of more than one IgE-binding epitope on the small fragment F16 (33 amino acids), demonstrating

both the highly individual IgE reactivity of the different patients' sera and the presence of multiple IgE-binding epitope regions distributed over the whole molecule.

Comparison with predicted B cell epitopes

For prediction of potential continuous antigenic determinants the amino acid sequence of rHol I 5.01 was analysed by two different computer programs for protein structure prediction (Fig. 4). For comparison the four small non-overlapping IgE-binding fragments F5, F10, F14 and F16 were aligned. These fragments covered six out of seven predicted epitopes. On fragment F16, on which we identified at least four IgE-binding epitopes, both methods predicted two potential antigenic determinants. The algorithms for fragment F14 also predicted more than one potential antibody binding site. Thus, further investigation of F14 might lead to the discovery of additional continuous IgE-binding epitope regions.

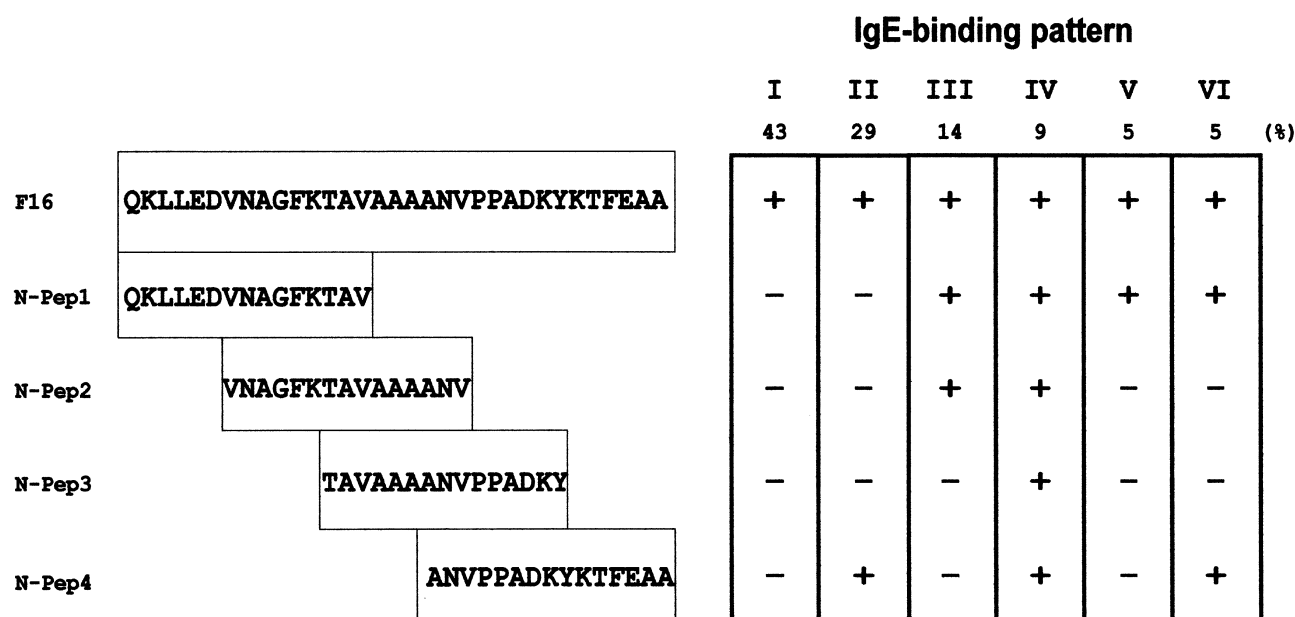


Fig. 3. Fine mapping of IgE-binding epitopes on the N-terminal fragment F16. Twenty-one patients' sera reactive to fragment F16 were mapped for their reactivity to four overlapping peptides (15 amino acids) (N-Pep1 to N-Pep4). Six different binding patterns (I to VI) were ordered according to their frequency of recognition given in percentage of investigated sera.

Inhibition experiments

Specificity of IgE binding to the rPhl p 5b and the fragments was shown by blot inhibition assays (Fig. 5). IgE binding to nHol 1 5 in velvet grass pollen extract blotted on nitrocellulose membrane was inhibited by MBP-Hol 1 5.01 and vice versa. IgE binding to MBP-fragments of rHol 1 5.01 was inhibited by complete MBP-Hol 1 5.01 while no inhibition was observed by using MBP alone. Binding affinity of the IgE antibodies to the complete rHol 1 5.01 and the fragment F16 was compared by *competitive* blot inhibition assay (Fig. 6). We incubated nitrocellulose strips with blotted MBP-F16 or MBP-F16 + MBP-Hol 1 5.01 with serum of individual F16-positive patients' sera. F16 was recognized by IgE on the strip with MBP-F16 only. On the strip containing MBP-F16 + MBP-Hol 1 5.01 IgE was quantitatively bound by rHol 1 5.01 and binding to F16 was completely inhibited, although 15-fold higher molar concentrations of F16 were used. Similar results were obtained for other small fragments (F5, F10, F14) (data not shown).

These results demonstrate that IgE antibodies binding to the small fragments were specifically directed against the complete rHol 1 5.01. On the other hand we observed that IgE reactivity to F16 and other small fragments was weaker than to the complete molecule (Fig. 3 and Fig. 6). Together with the finding that complete inhibition could be achieved by 15-fold reduced molar concentrations of the complete rHol 1 5.01 compared to F16, these results indicate that the

avidity of IgE antibodies for fragment F16 and other small fragments must be lower than that for the complete molecule.

Discussion

The systematic IgE-binding epitope mapping reported in this study revealed the presence of both continuous and discontinuous epitopes on rHol 1 5. The latter epitopes were destroyed by further fragmentation. Fine mapping with overlapping peptides of one of the small fragments, F16, revealed IgE reactivities to different parts of this fragment. Analysis of the antigenic structure of rHol 1 5 by prediction programs suggested that also on the fragment F14 such additional continuous epitope regions are present. The finding of multiple IgE-binding epitopes on one molecule is in line with the observation that molecule surfaces possess a variety of potential overlapping antigenic determinants dependent on intrinsic properties of the molecule, like accessibility and hydrophilicity [28,29]. However, this is the first report of such a high density of IgE-binding epitopes on one allergen molecule.

Different patients exhibited highly individual IgE reactivity to the different fragments of rHol 1 5.01. Longitudinal observation of individual patients over several years revealed that the binding pattern did not change and was characteristic for the individual patient. This highly individual IgE reactivity is possibly due to the genetic

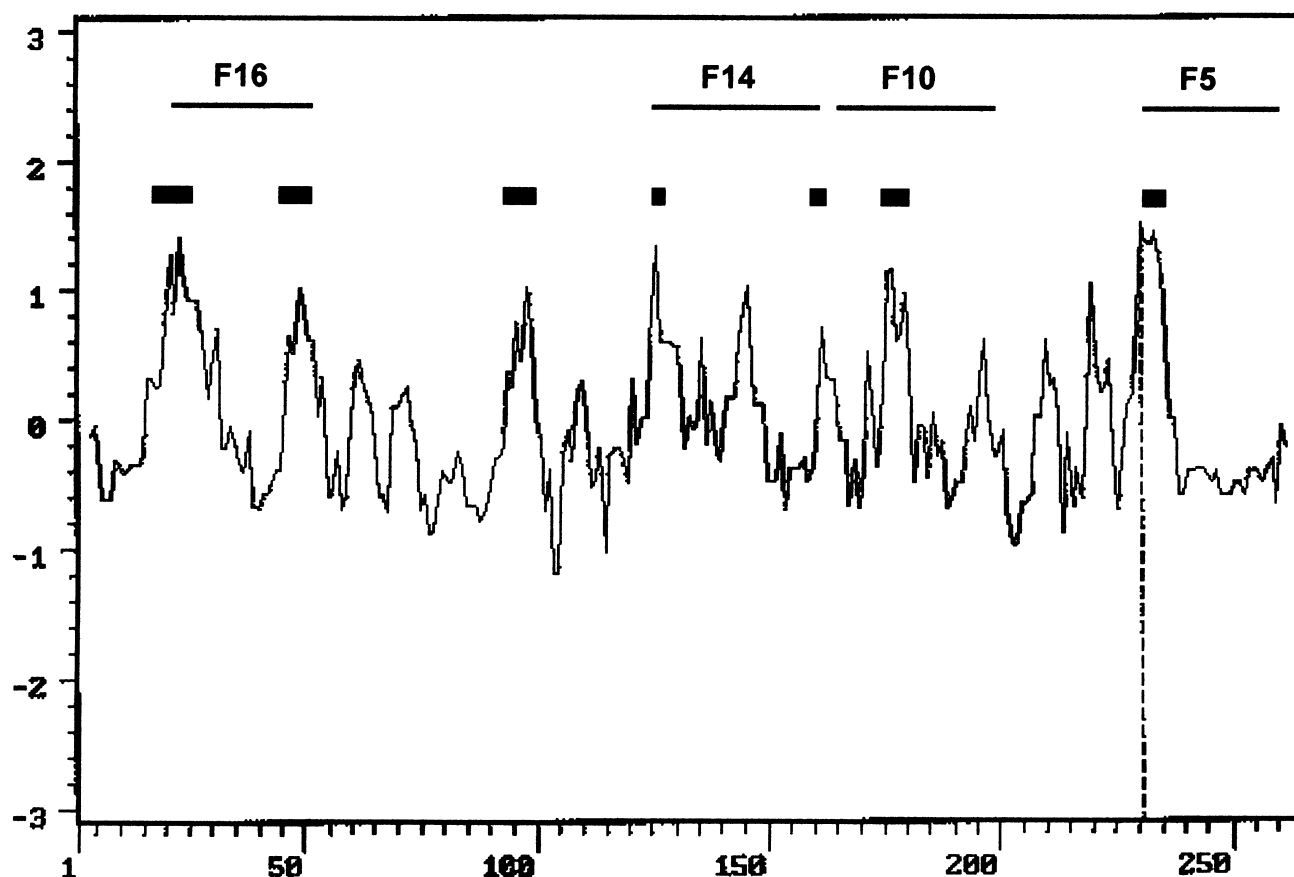


Fig. 4. Comparison of the identified IgE-binding fragments with predicted B cell epitopes. Antigenic determinants were predicted according to two methods: hydrophilicity profile according to Hopp and Woods (curve), and antigenic indices according to Jameson and Wolf. Sequence stretches with indices over 0.9 are shown as blocks. Identified IgE-reactive fragments are shown as solid bars.

background of the patient. Which of the antibodies are made by an individual depends on host factors such as self-tolerance, immune response genes (MHC), idiotype network and immunoglobulin structural gene repertoire [29]. For example, for mice strains genetically identical except for their MHC genes differential antibody reactivity to fragments of staphylococcal nuclease was observed [29,30].

IgE reactivity to small fragments was weaker than to larger fragments and often detectable only in sera with high specific IgE-titres. This observation might essentially have two reasons: (i) the concentration of IgE antibodies directed against one single epitope may be very low and close to the detection limit; (ii) epitopes on small peptides may not represent the complete correctly folded epitope but may be parts of larger discontinuous epitopes which are recognized by the same antibodies as the discontinuous epitopes but with lower affinity.

Blot inhibition experiments with the N-terminal fragment F16 confirmed the second possibility. In the presence of the complete rHol 1 5.01 molecule, IgE antibodies did not bind any longer to F16, even at 15-fold higher molar

concentrations. This demonstrates that antibodies binding to F16 are in fact Hol 1 5-specific antibodies and that the avidity of the IgE antibodies for F16 is lower than that for the complete rHol 1 5.01. Such comparatively low avidity of IgE antibodies was also reported for peptides of the major cat allergen Fel d 1 by Van Milligen *et al.* [31]. In contrast, on food allergens multiple IgE-binding epitopes were identified even on small synthetic peptides, indicating that in this case IgE antibodies bind to continuous epitopes with high avidity. On Ara h 1, a major peanut allergen, for example, 23 epitopes of 6–10 amino acids length were identified [13].

The reason for this contrasting observation might be the different biochemical processing of the molecules at their first encounter with the immune system. While food allergens are degraded to small fragments during their passage through the digestive tract, inhalational allergens such as pollen or house dust mite allergens are less exposed to enzymatic attack and, thus, may be able to penetrate the epithelial layer at full size. This may especially apply for small inhalational allergen molecules such as Bet v 1 or

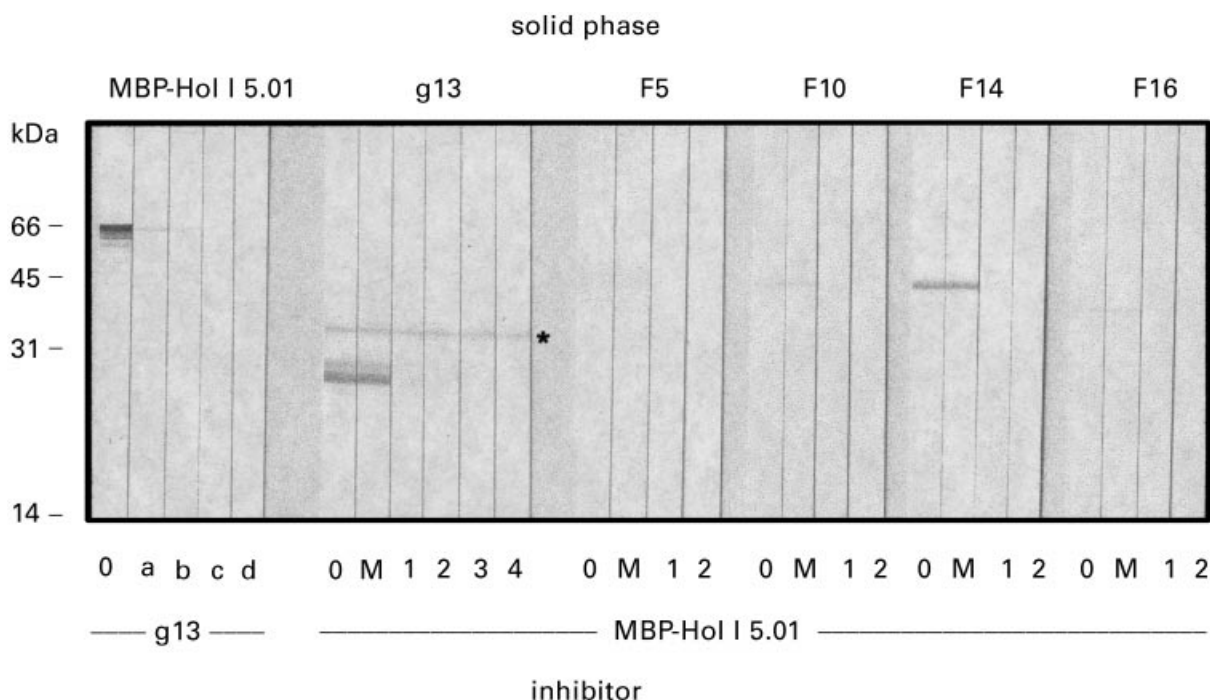


Fig. 5. Specificity of IgE binding to rHol I 5.01 and recombinant fragments shown by blot inhibition. Recombinant fusion protein MBP-Hol I 5.01 (1 $\mu\text{g}/\text{cm}$), velvet grass pollen extract (g13) (5 $\mu\text{g}/\text{cm}$), and recombinant MBP-fragments F5, F10, F14 and F16 (10 $\mu\text{g}/\text{cm}$) were separated by SDS-PAGE and blotted on nitrocellulose membrane (solid phase). Patients' sera were pre-incubated without (0) and with inhibitor g13 (a) 2, (b) 4, (c) 8, (d) 12 mg/mL serum or MBP-Hol I 5.01 (1) 20, (2) 40, (3) 80, (4) 800 $\mu\text{g}/\text{mL}$ serum. MBP alone was used as negative control at a concentration of 80 $\mu\text{g}/\text{mL}$ serum (M). The star marks the group I allergen, Hol I 1, which represents the second major allergen of velvet grass pollen.

Der p 2 which have a size of 18 kDa and 14 kDa, respectively. These molecules, which are found to possess mainly conformational epitopes [3,11], may be able to pass the mucosa undegraded. For larger molecules like the

30 kDa group V allergen isoforms of timothy grass pollen, Phl p 5a and b, Bufer *et al.* [32] reported that they were converted in the presence of nasal secretion to several defined fragments between 10 and 20 kDa.

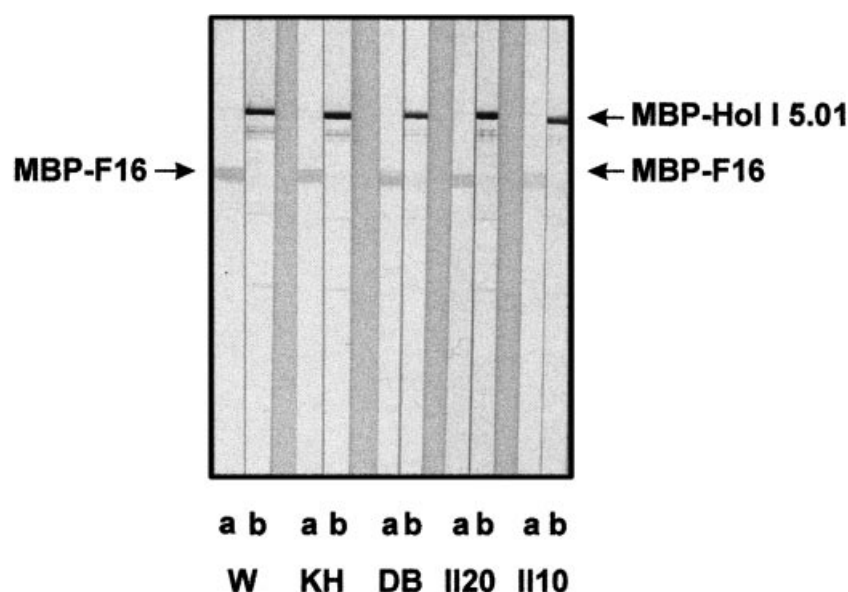


Fig. 6. Comparison of the affinity of IgE to fragment F16 and complete rHol I 5.01. MBP-F16 fusion protein (10 $\mu\text{g}/\text{cm}$) (a) and MBP-F16 (10 $\mu\text{g}/\text{cm}$) + MBP-Hol I 5.01 (1 $\mu\text{g}/\text{cm}$) (b) were separated by SDS-PAGE and analysed for IgE-binding by 5 different F16-reactive patients' sera (W, KH, DB, II20, and II10). Arrows indicate location of MBP-F16 and MBP-Hol I 5.01.

Interestingly, a stable N-terminal fragment of Phl p 5a and a stable C-terminal fragment of Phl p 5b exhibited even higher activity in histamine release and skin prick test than the complete molecule. These results correspond with our observation that the binding of IgE to the C-terminal fragment F2 is stronger than to the larger fragment F1 (Fig. 2), suggesting that it is not only the complete group V allergen but defined larger fragments (about 15 kDa) of these molecules which encounter the immune system and elicit the allergic response. Therefore, the route of immunization may influence the kind of epitopes recognized by IgE antibodies. From these data we suggest that the more the allergen is degraded the more the humoral immune response is directed to continuous epitopes.

In conclusion, on rHol 1.5 we identified multiple different continuous as well as discontinuous IgE-binding epitopes, which were individually recognized by IgE from different patients. However, IgE antibodies bound to small fragments with low avidity, indicating that the continuous epitope regions are parts of the larger discontinuous IgE-binding epitopes. Nevertheless, these mapping studies allowed us to identify parts of the molecule which are involved in antibody binding. The knowledge obtained from such studies can be useful for the development of allergen mutants with reduced IgE reactivity but retained T cell reactivity for improved immunotherapy [33].

Based on our epitope mapping results of rHol 1.5.01 reported in this paper, we constructed mutants of the homologous group V timothy grass pollen allergen rPhl p 5b by introducing specific deletions and were able to destroy most of the IgE reactivity as shown *in vitro* by blots, histamine release assays and *in vivo* by skin prick tests [34]. T cell reactivity with the deletion variants, however, was completely retained. Such engineered allergens might be applied at higher doses than the natural IgE-reactive allergens for effective immunomodulation, but without negative side-effects and therefore are promising candidates for a more efficient immunotherapy.

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