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A novel grass pollen allergen mimotope identified by phage display peptide library inhibits allergen–human IgE antibody interaction

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Abstract The aim of this study was to investigate the molecular basis of human IgE–allergen interaction by screening a phage-displayed peptide library with an allergen-specific human IgE-mimicking monoclonal antibody (mAb). A mAb that reacted with major grass pollen allergens was successfully identified and shown to inhibit human IgE–allergen interaction. Biopanning of a phage-displayed random peptide library with this mAb yielded a 12 amino acid long mimotope. A synthetic peptide based on this 12-mer mimotope inhibited mAb and human IgE binding to grass pollen extracts. Our results indicate that such synthetic peptide mimotopes of allergens have potential as novel therapeutic agents. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Grass pollen; Allergen; Epitope; Mimotope; Phage display; Human immunoglobulin E

1. Introduction

Immediate hypersensitivity reactions to certain foreign proteins, which can sometimes be fatal, are well known and documented [1]. This allergic response is due to histamine released from mast cells and basophils as a result of cross-linking of cell-bound immunoglobulin E (IgE) antibodies. Therefore, allergen-specific IgE antibodies serve as a valuable probe in allergen identification and characterisation. Using such antibodies, we and others have cloned and characterised a number of clinically significant allergens [2] and mapped some of their B cell [3] and T cell epitopes [4]. Further characterisation of the molecular basis of the allergen–IgE interactions may allow the design and production of synthetic haptens, based on such areas of interaction, that block IgE cross-linking and thus allow future development of novel therapeutics.

Allergy to grass pollen, which affects up to 25–30% of the population, has been reported frequently in the literature [5]. Among all grass types, rye grass pollen is the most clinically significant in temperate climates of the world producing up to half a tonne of pollen per hectare [6]. However, recent molecular and immunological studies have shown a high degree of cross-reactivity between taxonomically related grasses [2,7]. Such cross-reactivity is based on the occurrence of common IgE-binding epitopes among similar allergens in different grass pollens.

Current methods of allergy treatment are limited by safety and efficacy. For example, pharmacotherapy aims to neutralise the effects of the allergic reaction but is associated with a number of side-effects. On the other hand, allergen immunotherapy involves the periodic injection of allergen extracts, in increasing doses, which carries a risk of systemic and annoying local reactions. There is a need for alternative approaches to treatment. Allergen cross-reactivity not only simplifies diagnosis of grass pollen allergy but also could increase the efficacy of any potential therapeutic modalities that could be directed to be effective against several allergens.

One novel approach of investigating the molecular basis of allergen–IgE interactions uses phage display peptide library technology [8]. Phage-displayed peptide libraries allow for the surface display of myriads of different combinations of short stretches of amino acids (i.e. 7–12 residues in length) that can be screened with a target molecule. Phage that interact with the target molecule are then purified, amplified and subjected to several rounds of further selections referred to as 'biopanning' [8]. The short DNA insert that encodes the surface-displayed peptide is then sequenced and the amino acid sequence of the mimotope determined. In this way, allergen-specific IgE antibodies can be used as the target molecule to screen with the phage display peptide library to identify the allergen mimotope involved in the reaction. Once the amino acid sequence of the mimotope is determined, synthetic peptides can be produced and their effects as potential inhibitors of allergen–IgE interactions evaluated.

Although useful and easily accessible, naturally occurring human IgE antibodies are present in very low concentrations and are polyclonal in nature, making it difficult to use them as the target molecule to investigate such interactions. Monoclonal human IgE antibodies would be more desirable, but cannot be prepared easily due to difficulties in Epstein–Barr virus immortalisation of human B cells that produce IgE [9]. An alternative target molecule is an allergen-specific murine monoclonal antibody (mAb), obtained using hybridoma tech-

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; Ig, immunoglobulin; mAb, monoclonal antibody; NC, nitrocellulose; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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nology, that is immunologically similar to human IgE antibodies; that is, it recognises the same epitope as human IgE and therefore inhibits the allergen–IgE interaction. It has recently been reported that such a strategy has been successful with a murine mAb specific to the major house dust mite allergen Der p 1 [10].

We have identified a mAb that recognises major allergens of a number of clinically significant grass pollens. We investigated the ability of such a mAb to inhibit allergen–human IgE interactions. Once characterised, we defined the allergen mimotopes for this selecting mAb using a phage display peptide library. We studied the effect of such mimotopes on the allergen–mAb, and more importantly the allergen–human IgE interactions, using inhibition immunoassays. We report here the identification and immunological characterisation of the novel allergen mimotope using this technology.

2. Materials and methods

2.1. Pollen extracts

Dry and de-fatted *Lolium perenne* (perennial rye-grass), *Cynodon dactylon* (Bermuda grass), *Sorghum halepense* (Johnson grass), *Dactylis glomerata* (orchard grass), *Holcus lanatus* (velvet grass), *Poa pratensis* (Kentucky bluegrass), *Festuca elatior* (meadow fescue), *Phleum pratense* (timothy grass), and *Betula verrucosa* (birch) pollen were purchased from Greer Laboratories, Lenoir, NC, USA. Soluble proteins from these pollen were obtained and their protein concentrations determined as described elsewhere [11].

2.2. Patient sera

Human sera were obtained, with oral and written consent, from grass pollen-allergic and non-allergic individuals attending the Allergy Clinic at the Alfred Hospital. An allergic status was defined by a history of grass pollen allergy, positive skin prick test to grass pollen and the presence of serum IgE specific to grass pollen, as determined by radioallergosorbent test.

2.3. mAb production

Six-week-old BALB/c mice were each injected intraperitoneally with 50 μg of an equal mixture of total proteins from rye, Bermuda and timothy grass pollens in Hunter's Titermax adjuvant (CytRx, Athens, GA, USA). After 14 days, a booster injection of the same amount of protein mixture, without adjuvant, was administered. Ten days later, sera of the mice were collected by eye bleeding and tested for grass pollen reactivity via immunoblotting, as described below. Fifteen days after this, the final boosters were injected and after 3 days mice were killed. Their bloods were collected in 1.5-ml microfuge tubes and the sera stored at −20°C until required. Their spleens were dissected for use in generation of hybridomas following established techniques [12]. Hybridomas were screened against the grass pollen proteins by enzyme-linked immunosorbent assay (ELISA) as described below. Positive hybridomas were selected for expansion and cloning following standard methods [12]. Once cloned, the mAbs were tested by ELISA and confirmed by Western immunoblotting against a panel of grass pollen proteins. Since mAb 2A1 reacted with a number of allergens in different grass pollen extracts, it was re-cloned in order to ensure the monoclonal status of the cell line. mAb 2A1 was purified by protein G chromatography (Pierce, Rockford, IL, USA), following the manufacturer's instructions and used for biopanning and in inhibition immunoassays as described below.

2.4. Phage-displayed random peptide library screening

Purified mAb 2A1 (10 μg/50 μl/well in coating buffer) was immobilised onto the walls of microtitre plate wells as described for ELISA below. Biopanning of microtitre plate-coated mAb 2A1 with the PhD-12 phage display peptide library kit (New England BioLabs, Beverly, MA, USA) and characterisation of the binding clones was carried out following the manufacturer's instructions. Briefly, 1.4×10^{11} pfu of phage library (complexity = 1.9×10^{9} transforming units), was exposed to a plate coated with mAb 2A1, unbound phage were washed away and the specifically bound phage were eluted by lowering the pH. The eluted pool of phage was amplified and the process was repeated for a total of four rounds. After four rounds of biopanning, 10 individual clones were isolated and sequenced as described below.

2.5. Mimotope sequencing and peptide synthesis

Pure phage DNA was used for direct sequencing employing the [γ-^{32}P]ATP end-labelled primer protocol of the *fmol*™, PCR-based DNA sequencing system (Promega, Madison, WI, USA) according to the manufacturer's instructions. After the PCR sequencing reactions were completed, the samples were heated at 75–80°C for 2 min and loaded onto a 6% polyacrylamide/7 M urea gel (Sequagel, Kimberly Research, NJ, USA) and the nucleotides were separated by electrophoresis at a constant power of 40 W. The gel was transferred onto Whatman 3 mm paper (Whatman, Maidstone, UK), dried at 80°C under vacuum and exposed to X-ray film (XAR, Kodak) overnight at −70°C.

Peptide, based on the amino acid sequence of the mimotope, was synthesised commercially using Fmoc chemistry and purified by high pressure liquid chromatography (Auspep, Vic, Australia).

2.6. Mimotope sequence analysis

Amino acid sequences were deduced from the DNA sequences and searched for comparison with other sequences in the Australian National Genomic Information Service (ANGIS) database, which incorporates databases from the following major sources: GenBank, EMBL, NBRF PIR protein and SwissProt libraries.

The frequency of amino acids present in the 10 peptides was compared by χ^2 test with the frequency of amino acids in peptides derived from the naive unpanned library as listed by the supplier (New England BioLabs). The sequences of the peptides were aligned with each other using the program PILEUP in conjunction with the Tudos matrix which compares amino acids on the basis of physicochemical properties [13,14]. Since the peptides are expressed at the amino-terminus of pIII and therefore are structurally flexible, a low penalty for the introduction of gaps was used. The peptides were then aligned as a group with the sequences of the rye grass pollen allergens Lol p 1, Lol p 2 and Lol p 5 to reveal patches of similarity with the target sequences.

2.7. Gel electrophoresis

Total grass pollen proteins (30 μg/lane) and pre-stained molecular weight markers (Life Technologies, USA; 10 μl/lane) were resolved on 12.5% 'mini' sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels in a Xcell II Mini-Cell (Novex, CA, USA), following the manufacturer's instructions and as described previously [11,15].

2.8. Western immunoblotting

SDS–PAGE gels containing total proteins of different grass pollens were transferred onto nitrocellulose (NC) membranes (BA 0.45 μm, Schleicher and Schuell, Dassel, Germany) using Xcell II blotting apparatus (Novex), following the manufacturer's instructions. Following immobilisation of proteins onto NC membranes, blots were blocked in 10% w/v skim milk powder in phosphate-buffered saline (PBS) and probed with either mAb 2A1 (cell culture supernatant diluted 1:9, 5 ml/blot) or human sera containing IgE (serum pool of three grass pollen-allergic individuals diluted 1:4, 5 ml/blot) following established protocols [11]. Briefly, the secondary antibody for detecting mAb 2A1 binding was horseradish peroxidase (HRP)-conjugated anti-mouse Igs (Silenus, Australia) while human IgE binding was detected with rabbit anti-human IgE (Dako, Denmark) followed by HRP-conjugated anti-rabbit Ig (Promega). Hydrogen peroxide and the chromogen 4-chloro-1-naphthol (Sigma, St. Louis, MO, USA) were used to visualise the antibody binding sites on the blots [11]. All blots were incubated together for up to 30 min to ensure relative development between the different blots. Negative control blots were probed with a serum pool of three non-allergic individuals while 'no-serum/mAb' controls were probed with the secondary and/or tertiary detection antibodies only. Negative control blots failed to demonstrate any antibody binding (data not shown).

2.9. ELISA

Total rye grass pollen proteins (10 μg/well) in 50 μl/well of coating buffer (60 mM sodium bicarbonate/30 mM sodium carbonate, pH 9.3) were used to coat ELISA microtitre plates at 4°C overnight. After

washing wells 10 times with 0.1% Tween 20 in PBS, the wells were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. After washing of all wells, the coated wells were incubated at 37°C for 1 h with 50 μl/well with either human serum (diluted 1:4) or mAb 2A1 (neat supernatant or diluted 1:9). After extensive washing, human serum wells were incubated with 50 μl/well of rabbit Ig to human IgE (Dakopatts, CA, USA; diluted 1:750 in PBS/BSA) followed by peroxidase-conjugated anti-rabbit Ig (Promega; diluted 1:2500 in PBS/BSA) while mAb 2A1 wells were incubated with 50 μl/well of peroxidase-conjugated anti-mouse Ig (Amrad, Australia; diluted 1:500) for 1 h each at 37°C, with extensive washing of all wells at the end of each step. The plates were then incubated with 50 μl/well of peroxidase substrate (0.00024% w/v *o*-phenylenediamine, 0.0006% H_2O_2 in distilled water) for 10 min at 37°C and the reaction was stopped with 25 μl/well of 4 N H_2SO_4. The colour reaction was then read at OD 492 nm. Negative control wells were incubated either with a serum pool of three non-allergic individuals or with the secondary and/or tertiary detection antibodies only (blank). Negative controls failed to demonstrate any significant antibody binding and these values were deducted from the test values (data not shown).

2.10. Inhibition immunoblot assay

Inhibition studies were conducted with NC blots, containing SDS–PAGE-resolved total pollen proteins from a panel of clinically significant grasses, incubated either with a pool of sera from three grass pollen-allergic patients or mAb 2A1 in the absence (positive control) or pre-incubated for 1 h at room temperature in the presence of potential inhibitors of human IgE/mAb 2A1 binding. In order to determine the ideal serum and mAb 2A1 dilution where antibodies were neither in excess or limiting, individual NC strips of SDS–PAGE-resolved total rye grass pollen proteins were incubated with the serum pool and mAb 2A1 supernatant at a number of dilutions. The optimal dilutions were 1:4 for human serum and 1:9 for mAb 2A1. Inhibitors included the 12 amino acid long synthetic peptide (based on the mimotope sequence, see below; 500 μg/5 ml/blot) or crude rye grass pollen extract (150 μg/5 ml/blot). Detection of antibody binding and inclusion of negative controls was the same as that described above.

2.11. Inhibition ELISA

The method was essentially the same as for the direct ELISA described above. However, the rye grass pollen protein-coated wells were incubated at 37°C for 1 h with 50 μl/well with either dilution of serum or mAb 2A1 that had been pre-incubated with different amounts of potential inhibitors for 1 h at room temperature. Inhibitors included different amounts of either purified mAb 2A1 (to assess the affect of mAb 2A1 on IgE binding to rye grass pollen extract) or sera (to assess the effect of IgE on mAb 2A1 binding to rye grass pollen extract) in a total volume of 50 μl/well. Other inhibitors included synthetic peptide (50 μg/well) based on the mimotope sequence (to assess its effect on human IgE/mAb 2A1 binding to rye grass pollen extract) and rye grass pollen extract (50 μg/well) as positive control. Before embarking on these assays, dilution curves for both the human sera and mAb 2A1 were conducted to determine optimal dilutions at which the antibodies were neither in excess nor limiting. The optimal dilutions were 1:200 for human serum and 1:400 for mAb 2A1. Detection of antibody binding and inclusion of negative controls (blank) was the same as described for ELISA above. Percentage inhibition was calculated by the following formula:

$$\%\ \text{Inhibition} = 100 - \left(\frac{\text{Absorbance value with inhibitor}}{\text{Absorbance value without inhibitor}} \times 100\right)$$

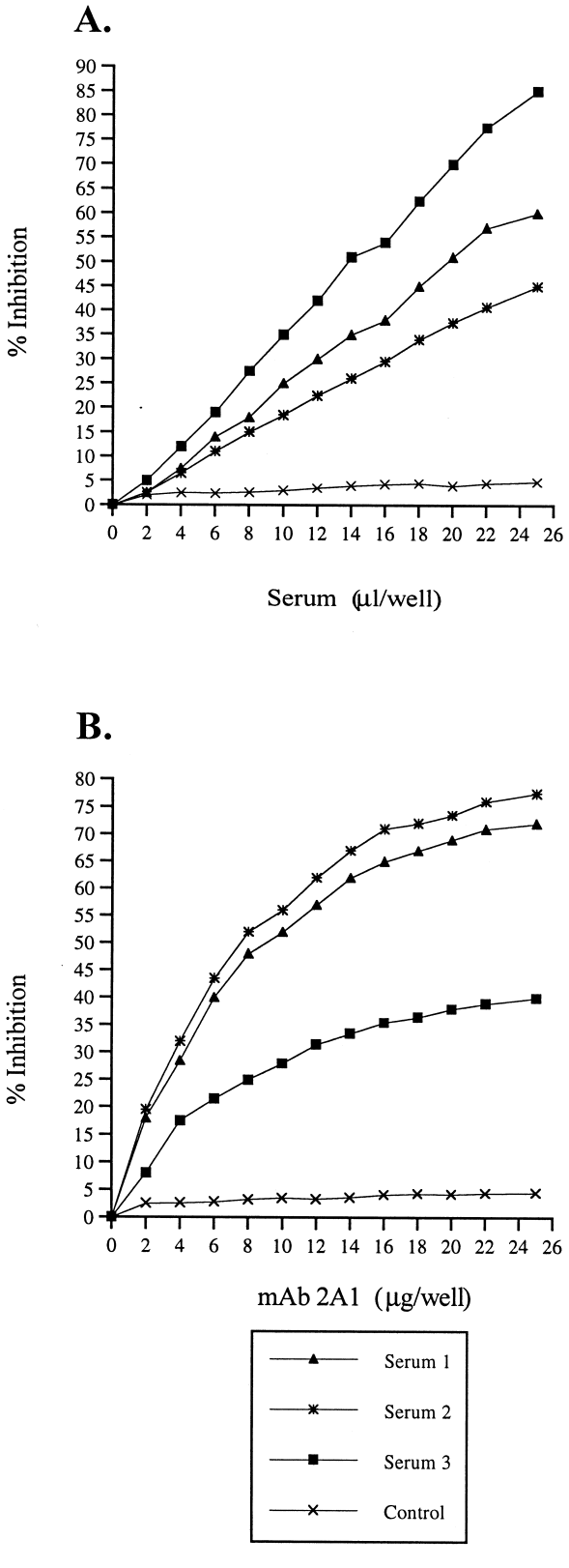

Fig. 1. Inhibition of mAb 2A1 reactivity by sera from atopic individuals. A: Inhibition of mAb 2A1 binding to total proteins of rye grass pollen by individual patient sera. Percentage inhibition of mAb 2A1 (diluted 1:400) is shown on the *y*-axis and the amount (μl/well) of neat individual serum, used as the potential inhibitor, is shown on the *x*-axis. A serum pool of three non-atopic individuals was used as a negative control. B: Inhibition of human serum IgE binding to total proteins of rye grass pollen by mAb 2A1. Percentage inhibition of human serum IgE (diluted 1:200) is shown on the *y*-axis and the amount (μg/well) of mAb 2A1, used as the potential inhibitor, is shown on the *x*-axis. The effect of mAb 2A1 on IgE binding, from the serum pool of three non-atopic individuals, was used as a negative control.

3. Results

3.1. Inhibition ELISA characterisation of mAb 2A1

Among 10 mice injected with equal amounts of Bermuda, rye and timothy grass pollen extracts, a test bleed of one mouse demonstrated reactivity to several grass pollens of clinical significance. This mouse was chosen to clone a mAb-producing hybridoma cell line, 2A1, that recognised the panel of clinically significant grass pollen allergens.

The mAb 2A1 was purified and used in a number of immunological studies to characterise its specificity. We first wanted to establish whether mAb 2A1 recognises the same epitope(s) as human IgE on the different grass pollen allergens. Therefore, we performed inhibition ELISAs to assess whether serum of grass pollen allergic patients can inhibit mAb 2A1 binding to rye grass pollen extract. Among three atopic individuals, significant inhibition of mAb 2A1 binding occurred, while no significant inhibition was observed when a serum pool from three non-atopic negative controls was used (Fig. 1A). Conversely, we assessed the capacity of mAb 2A1 to inhibit human IgE binding to rye grass pollen allergens. Once again, we showed that mAb 2A1 can significantly inhibit human IgE binding to rye grass pollen allergens among the same three grass pollen-allergic patients (Fig. 1B). The highest inhibition of IgE binding to rye grass pollen extract by mAb 2A1 was observed with serum of subject 2, followed by 1 and 3 (Fig. 1B).

3.2. Biopanning of a phage-displayed peptide library and mimotope identification of mAb 2A1

As a result of mAb 2A1's capacity to inhibit human IgE binding to rye grass pollen extracts, we next wanted to identify the allergen mimotope that was responsible for both mAb

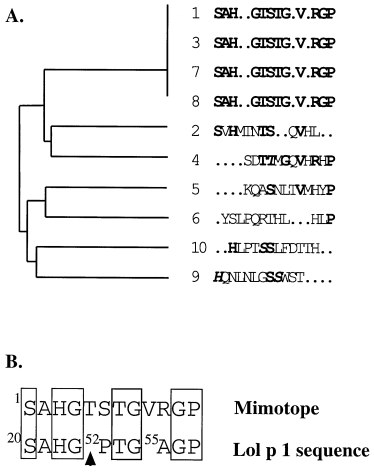

Fig. 2. Mimotope sequence analysis. A: Alignment and sequence of phage-displayed peptides selected by the mAb 2A1. The sequences of the peptides were subjected to the multiple alignment algorithm PILEUP which produces a guide tree indicating the degree of similarity of the peptides to each other and aligns the sequences. The residues that were identical to those in the frequently selected peptide are in bold and conservatively substituted residues are in bold italics. B: Alignment of the mimotope sequence with the primary sequence of Lol p 1. The positions of Lol p 1 with which residues of the mimotope sequence align are given in superscript and the amino acids that were specifically selected for by the mAb 2A1 are boxed. The arrow indicates insertion of a gap in the sequence of Lol p 1 to optimise the alignment by PILEUP.

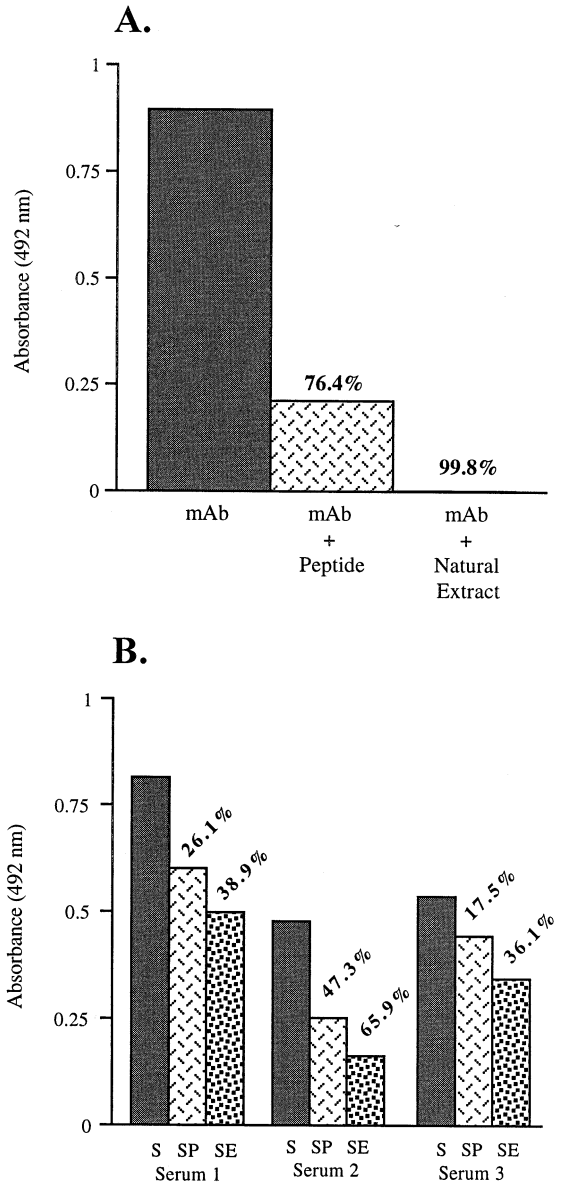

Fig. 3. Inhibition of mAb and serum IgE reactivity by the peptide mimotope. A: Inhibition of mAb 2A1 binding to total proteins of rye grass pollen by synthetic peptide mimotope and total rye grass pollen proteins. Absorbance (at 492 nm) for antibody binding is shown on the *y*-axis and the antibody binding in the absence (left bar) and presence of the inhibitors synthetic peptide mimotope (middle bar) and total rye grass pollen proteins (right bar) is shown on the *x*-axis. Percentage inhibition of mAb binding by the respective inhibitors is indicated by percentage figures on the bars. Omission of the mAb 2A1 incubation step was included as a negative control and deducted from the final readings. B: Inhibition of human serum IgE binding to total proteins of rye grass pollen by synthetic peptide mimotope and total rye grass pollen proteins (used as a positive control). Absorbance (at 492 nm) for antibody binding is shown on the *y*-axis and the antibody binding in the absence (S; left bars) and presence of the inhibitors synthetic peptide mimotope (SP; middle bars) and total rye grass pollen proteins (SE; right bars) is shown on the *x*-axis for each of the three individual sera. Percentage inhibition of human IgE binding by the respective inhibitors is indicated by percentage figures on the bars. Omission of the serum incubation step was included as a negative control and deducted from the final readings.

2A1 and human IgE binding. Identification of such a mimotope could then facilitate the production of synthetic mimotopes that can be used to further investigate inhibition of mAb 2A1 and human IgE binding to rye grass pollen extract.

After biopanning, 10 individual clones were randomly chosen and the DNA insert sequenced. The deduced amino acid sequences of the 10 clones are given in Fig. 2. Four of the clones shared 100% amino acid sequence identity with each other over the 12-amino acid peptide length (Fig. 2A). The remaining six clones contained some but not all of the amino acid residues.

3.3. Mimotope sequence analysis

Database comparison of the amino acid sequences of each clone did not reveal any significant homologies to known allergens. Comparison of the frequency of amino acids in the sequences of the 10 peptides derived from the phage-displayed library by the mAb 2A1 with those of peptides from the naive library reveals that the hydroxyl-containing residues (serine and threonine; $P = 0.0154$), glycine ($P < 0.001$) and histidine ($P = 0.0116$) were specifically selected for biopanning with 2A1. This indicates that these amino acids are likely to participate in forming the epitope for 2A1. Alignment of the sequence of the most frequently selected mimotope with the sequence of Lol p 1 showed that the first four residues of the mimotope are identical to amino acids 20–24 of Lol p 1 whilst residues 7–12 of the mimotope contain four identical residues to amino acids 53–57 of Lol p 1 (Fig. 2B). Meaningful alignments with other allergens (i.e. Lol p 5) were not found. Thus, whilst there was no similarity found between the mimotope and continuous stretches of amino acids of allergens present in the protein database, it is likely that the mimotope represents a conformational epitope of group 1 allergens, including Lol p 1, that is formed by the juxtaposition on the surface of amino acids of Lol p 1 from 20–24 and 53–57, in particular serine, threonine, glycine and histidine. Indeed, Lol p 1 (20–24) and Lol p 1 (53–57) sequences are conserved among other group 1 grass pollen allergens. Therefore, this peptide was chosen for further immunological studies as outlined below.

3.4. Inhibition ELISA characterisation of synthetic peptide mimotope

A 12-mer peptide, based on the mimotope amino acid sequence (i.e. SAHGTSTGVRGP), was synthesised and used as an inhibitor in inhibition ELISA immunoassays to assess its capacity to inhibit mAb 2A1 and human IgE binding to rye grass pollen extract. When compared with mAb 2A1 binding in the absence of an inhibitor, pre-incubation of the same amount of mAb 2A1 with 50 μg of mimotope peptide showed 76.4% inhibition (Fig. 3A), suggesting that this mimotope sequence functionally mimics the natural epitope recognised by the mAb 2A1. Pre-incubation of mAb 2A1 with 50 μg of total rye grass pollen extract (as a positive control) gave 99.8% inhibition (Fig. 3A).

When sera of the three atopic patients were incubated with 50 μg of either the mimotope peptide or total rye grass pollen extract and compared to total IgE binding in the absence of an inhibitor, the highest inhibition with both the mimotope peptide (47.3%) and the rye grass pollen extract (65.9%) was observed with serum of subject 2 (Fig. 3B), which correlates highly with the earlier inhibition studies using mAb 2A1 as the inhibitor (Fig. 1B). The next highest inhibition of IgE binding to rye grass pollen extract by the mimotope peptide was observed with serum of subject 1 followed by 3 (Fig. 3B), once again correlating with earlier results (Fig. 1B). Thus, the mimotope represents an epitope that is recognised by human IgE from allergic subjects.

We also tested synthetic peptides corresponding to the re-

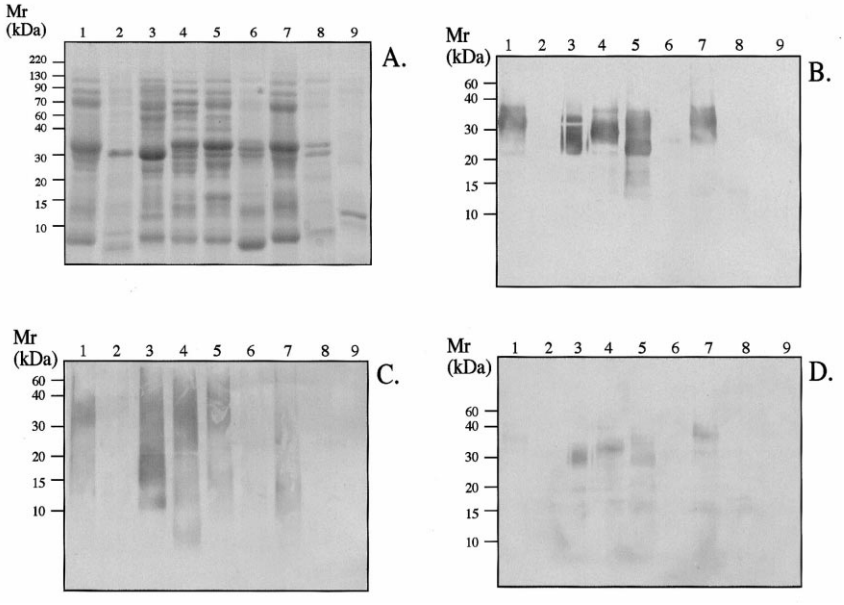

Fig. 4. Inhibition immunoblot analysis of mAb 2A1 binding to grass and birch pollen extracts. A: SDS–PAGE-resolved protein profiles of clinically significant grass pollens and birch pollen (used as a negative control) stained with Coomassie brilliant blue. mAb 2A1 binding in the absence of inhibitors (B) or in the presence of synthetic peptide mimotope (500 μg) (C) or total rye grass pollen proteins (150 μg) (D). Molecular mass markers (M) are indicated on the left in kDa. Different lanes contain total proteins from rye grass (lane 1), Bermuda grass (lane 2), Johnson grass (lane 3), orchard grass (lane 4), velvet grass (lane 5), Kentucky bluegrass (lane 6), meadow fescue (lane 7), timothy grass (lane 8) and birch pollens (lane 9).

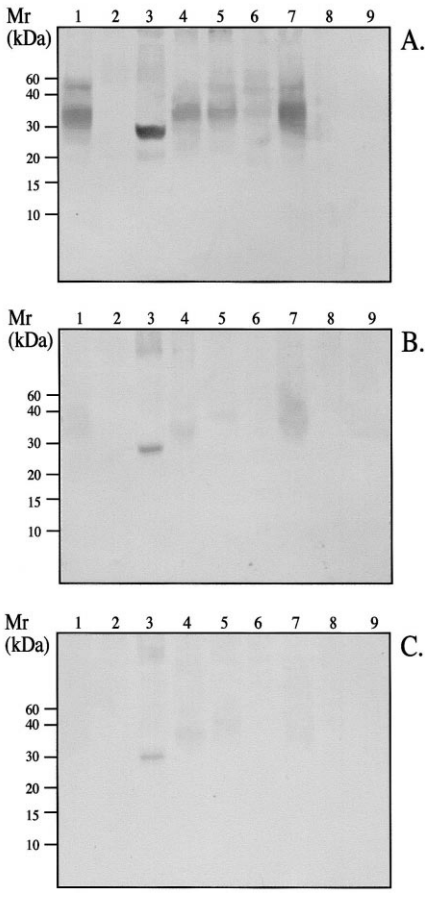

Fig. 5. Inhibition immunoblot analysis of human IgE binding to grass and birch pollen extracts. Pooled human serum IgE binding in the absence of inhibitors (A) or in the presence of synthetic peptide mimotope (500 μg) (B) or total rye grass pollen proteins (150 μg) (C). Lanes 1–9 are the same as described in Fig. 4.

maining six mimotope sequences (Fig. 2A) by direct ELISA using ELISA plate-bound peptides. Human IgE binding was only detected with the peptide corresponding to mimotope 1 (Fig. 2A) with sera of all three atopic patients while no binding was detected with the serum pool of non-atopic patients (data not shown).

3.5. *Inhibition immunoblot characterisation of synthetic peptide mimotope*

In order to assess and visualise the inhibition of mAb 2A1 and human IgE binding, by the mimotope peptide, to allergens of eight grass and one tree pollens, we used an inhibition immunoblotting assay. Fig. 4A shows the protein profile of the nine clinically significant pollens. Although we aimed to load the exact amounts of crude pollen proteins in each well, some samples may look a little faint due to their concentration being limited by the volume capacity of the gel sample well. In the absence of inhibitors, mAb 2A1 recognises a number of proteins of several grasses belonging to group 1 and possibly to group 5 allergens based on the specificity of group 1- and group 5-specific mAbs (data not shown) and their molecular masses (Fig. 4B). On the other hand, pre-incubation of mAb 2A1 with the mimotope peptide causes significant inhibition to the same proteins (Fig. 4C). It is interesting to note that in Fig. 4C, broad bands appear after incubation of mAb 2A1 with the mimotope peptide. The most likely reason for this may be the long development time (in order to ensure that inhibition has occurred) contributing to the high background development and therefore smearing or fusing the specific bands together. Pre-incubation of mAb 2A1 with rye grass pollen extract (positive control) completely inhibits antibody binding not only to rye grass pollen extract but also to the other grass pollen proteins, further highlighting the presence of shared epitope specificity among different grass pollens (Fig. 4D). Incubation of a replicate blot with detection antibodies only (no-mAb 2A1 negative control) did not show any antibody binding (data not shown).

In a similar experiment looking at the inhibition of human IgE antibody binding (using a serum pool of the three atopic patients), total IgE binding to the same proteins (Fig. 5A) is significantly inhibited by the mimotope peptide (Fig. 5B). It is interesting to note that in inhibition ELISA the inhibition was relatively partial even in the presence of 1 mg/ml of the inhibitor (Fig. 3B) while the immunoblotting was almost completely inhibited by 150 μg/ml (Fig. 5B). A possible reason for this observation may be that both the conformation epitope-specific antibodies and primary sequence epitope-specific antibodies can be detected in ELISA while only the latter antibodies can be detected in Western blotting analysis. Total rye grass pollen extract nearly completely inhibited total IgE binding (Fig. 5C). Incubation of replicate blots with either serum from a non-atopic patient or detection antibodies only (no-serum negative control) did not show any antibody binding (data not shown). These results further confirm that the identified mimotope binds both mAb 2A1 and human IgE and the epitope it represents occurs among related allergens of different grass pollens. Moreover, as a result of successful inhibition to natural proteins, the mimicked epitope appears to be surface-exposed and occur as a discontinuous epitope within the context of the natural proteins.

4. Discussion

One of the most critical factors of type I allergic responses is cross-linking of IgE antibodies on basophils and mast cells, leading to degranulation and release of chemical mediators. A better understanding of the molecular basis of human IgE–allergen interaction could lead to new ways to produce synthetic molecules as 'blockers' which may be beneficial for allergy treatment.

As a result of cloning and primary structure determination, B and T cell epitopes have already been mapped for many major allergens. However, no major advances in allergy treatment have resulted from such work. On the other hand, although there has been great interest, very little work has been reported on the cloning and characterisation of the epitopes recognised by human IgE mAbs. With advances in recombinant DNA technology, allergen-specific human mAbs have been studied recently using phage display technology [16]. A lack of cross-reactivity has been observed between the native and recombinant Fab of human IgE [16].

Another attractive approach is to produce allergen-specific murine mAbs that recognise the same epitopes as human IgE and therefore inhibit human IgE–allergen interaction. Such mAbs could in principle be used on their own for allergy treatment, but studies have shown that non-humanised mur-

ine antibodies are incompatible with the human immune system [17]. A great deal of research and resources has been put into humanising of such antibodies [17]. An alternative approach is to identify epitopes for such mAbs and investigate if these inhibit human IgE–allergen interaction. We have obtained such an allergen-specific mAb and report here the identification of a mimotope of its epitope using a phage-displayed peptide library.

The allergen-specific mAb 2A1 inhibits human IgE binding to rye grass pollen extract, and human IgE inhibits mAb 2A1 binding to the rye grass pollen extract. This demonstrates the presence of conserved epitope specificity between human IgE and mAb 2A1. Using a phage-displayed peptide library, we screened mAb 2A1 to identify allergen mimotopes, and identified a 12-mer motif which occurs in 40% of the phage clones sequenced. Screening of the amino acid sequence against the protein database revealed no significant linear identity with any allergens sequenced to date. However, alignment of the sequence of the mimotope with sequenced allergens of rye grass pollen revealed patches of identity between parts of the mimotope and discontinuous regions of Lol p 1 and these regions of Lol p 1 were conserved among other group 1 grass pollen allergens. Thus, the phage-displayed peptide may represent a discontinuous, surface-exposed epitope comprising amino acids from different regions of the primary sequence that are brought together by the particular folding of the natural protein. We have recently reported that it is possible for phage-displayed peptides selected by a particular antibody to mimic discontinuous epitopes that are dependent upon protein conformation [18].

A peptide based on the 12-mer mimotope was synthesised and shown to inhibit binding of both mAb 2A1 and human IgE to rye grass pollen extract. This highlights that the identified mimotope corresponds to an epitope recognised by both mAb 2A1 and human IgE from atopic individuals. In order to assess if such a mimotope peptide inhibits mAb 2A1 and human IgE binding to clinically significant grass and tree pollens, we performed inhibition immunoblotting assays. Significant mAb 2A1 and human IgE binding were observed with most of the pollens and with several of the major allergens of these pollens. Although there is significant evidence for the existence of allergen homologues in different grass pollens [7], this highlights the conservation of this 12-mer epitope among similar allergens present in the different pollens.

The presence of such similar allergenic epitopes may simplify both diagnosis and treatment. A cross-reacting range of mimotopes may be sufficient for diagnosis. It is now important to assess the efficacy of the peptide we have described in a population of grass pollen-allergic patients.

Recently another group reported the use of a phage display peptide library to screen a house dust mite (Der p 1) allergen-specific murine mAb, which was shown to inhibit human IgE–Der p 1 interaction [10]. As in our studies, the interaction between the mAb and Der p 1 can be blocked by the mAb-specific phage clones. In contrast to our data, inhibition of human IgE binding to Der p 1 was not demonstrated with the identified mimotope [10]. Also, phage clones displaying the peptide mimotope were used, rather than the synthetic peptide molecule. Since the three-dimensional model of Der p 1 has previously been published [10], the authors could successfully locate the phage-displayed mimotope on the surface of Der p 1 by automated comparative modelling [10]. Unfortunately, no such three-dimensional models exist for grass pollen allergens that would allow us to locate the phage-displayed mimotope. Judging from the Der p 1 studies above, and the homology with patches of Lol p 1, the mimotope identified here may also be surface-exposed on grass pollen allergens. A comparison of three-dimensional structures of other cysteine proteases (i.e. chymopapain, papain and actinidin, which are also recognised by the Der p 1-specific mAb) revealed similar motif sequences, highlighting the importance of structure-based immunological cross-reactivity [10].

This is the first report that a synthetic peptide of a grass pollen mimotope blocks the allergen–mAb interaction and also allergen–human IgE interaction. Current efforts are now directed at the detailed analysis of reactivity and dose–response inhibitory capacity of the peptide mimotope among larger atopic and non-atopic patient groups. Moreover, it would be important to assess if the peptide induces allergic reactions in vivo (i.e. basophil histamine release). Such collective novel information may lead to new ways to block allergen–human IgE interactions to provide specific therapy of allergic diseases.

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