Construction of a Combinatorial IgE Library from an Allergic Patient

ISOLATION AND CHARACTERIZATION OF HUMAN IgE Fabs WITH SPECIFICITY FOR THE MAJOR TIMOTHY GRASS POLLEN ALLERGEN, Phl p 5

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To characterize human IgE antibodies with specificity for a major allergen at the molecular level, we have constructed an IgE combinatorial library from a grass pollen allergic patient. cDNAs coding for IgE heavy chain fragments and for light chains were reverse-transcribed and polymerase chain reaction-amplified from RNA of peripheral blood lymphocytes and randomly combined in plasmid pComb3H to yield a combinatorial library of 5 x 10^6 primary clones. IgE Fabs with specificity for Phl p 5, a major timothy grass pollen allergen, were isolated by panning. Sequence analysis showed that the 4 of the Fabs used the same heavy chain fragments which had combined with different kappa light chains. Soluble recombinant IgE Fabs were purified by affinity chromatography to Phl p 5 and, like natural IgE antibodies, cross-reacted with group 5 allergens from different grass species. The described approach should facilitate studies on the molecular interaction between IgE antibodies and allergens and encourages the consideration of specific IgE Fabs that are capable of interfering with allergen-IgE binding as potential therapeutic tools.

More than 20% of the population suffers from type I allergic reactions (allergic rhinitis, conjunctivitis, and bronchial asthma). The symptoms of type I allergy are due to release of mediators (e.g. histamine) resulting from the cross-linking of specific IgE antibodies, which are bound to allergic effector cells (mast cells and basophils). Studies on the primary structure of immunoglobulin E were initially hampered by the extremely low concentration of IgE (10^-40 ng/ml) in the serum. Due to the availability of IgE-secreting myeloma cells, it was, however, possible to characterize IgE antibodies by immunoechemical, protein chemical, and finally molecular biological techniques (Bennich et al., 1968, 1973; Ishizaka and Ishizaka, 1970; Terry et al., 1970; Kochwa et al., 1971; Flanagan and Rabbitts, 1982; Kurokawa et al., 1983; Seno et al., 1983). The cDNA sequence of human Cₑ could be determined (Flanagan and Rabbitts, 1982; Kurokawa et al., 1983; Seno et al., 1983), and those portions of Cₑ that interact with the high affinity Fc receptor were characterized as possible targets for a therapy of Type I allergic diseases (Helm et al., 1988; Nissim and Eshar, 1992; Presta et al., 1994).

To investigate the molecular interaction of IgE antibodies and allergens, studies on the V regions of specific IgE antibodies would be needed. Because of the low number of IgE-secreting B-cells in the peripheral blood of allergic patients (McKenzie and Dosch, 1989), a detailed study of allergen-specific IgE antibodies and in particular of their V regions has proven to be extremely difficult. In addition, it has been far impossible to immortalize B-lymphocytes that were switched to specific IgE production in vivo.

Using PCR techniques, nucleotide sequences of epsilon VH₂, transcripts from peripheral blood B-cells of atopic patients were analyzed, suggesting that the molecular characteristics of the ε VH₂ regions argue for a selection process due to recurrent or chronic stimulation of the immune system by antigens (e.g. allergens), but nothing is known about their specificities (van der Stoep et al., 1993).

In the present study, the isolation and characterization of human IgE Fabs with specificity for a major allergen is reported. For this purpose we have constructed an IgE combinatorial library from blood lymphocytes of a grass pollen allergic patient by reverse transcription and PCR amplification of cDNAs coding for IgE Fd and L chains. The cDNAs were randomly combined in the pComb3H vector (Barbas et al., 1991; Kang et al., 1991a) and expressed on the surface of filamentous phage to allow the selection of IgE Fab-expressing phage clones by panning to given allergens. Purified recombinant timothy grass pollen allergens Phl p 1 (Laffer et al., 1994), Phl p 2 (Dolecek et al., 1993), and Phl p 5 (Vrtale et al., 1993a) were used to determine the IgE specificities of the allergic patient. Recombinant human IgE Fabs with specificity for Phl p 5, a major timothy grass pollen allergen (Vrtale et al., 1993a), were isolated by panning and analyzed.

The present approach may contribute to the molecular analysis of allergen-IgE interactions and may be perhaps be useful to define recombinant Fabs, which, due to the lack of the Fc receptor binding site, may be envisaged as potential therapeutic tools that can compete with natural IgE antibodies for the allergen binding.

EXPERIMENTAL PROCEDURES

Characterization of the Grass Pollen Allergic Patient—For the construction of the IgE combinatorial library, peripheral blood mononuclear cells were obtained during the grass pollen season from a grass pollen allergic patient after informed consent was given. The patient

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The abbreviations used are: PCR, polymerase chain reaction; ELISA, enzyme-linked immunosobent assay; PAGE, polyacrylamide gel electrophoresis; CDR, complementarity determining region; Fds, heavy chain fragments.

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The nucleotide sequences reported in this paper have been submitted to the GenBank/European Molecular Biology Laboratory Data Bank with accession number(s) X95746–X95750.

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suffered from allergic conjunctivitis and rhinitis during the grass pollen season, was skin test-positive for timothy grass, and had received no hyposensitization treatment. Total serum IgE was determined by radioimmunoassay with a RIACT kit (Pharmacia, Uppsala, Sweden) to be 5,000,000 units/liter and a RAST class of >4 for timothy grass pollen was measured. The patient was further tested for IgE reactivity to purified recombinant allergens (B. c. 1 and B. c. 11) (Brehm et al., 1991; Valenta et al., 1991) and timothy grass pollen allergens (Phl p 1, Phl p 2, and Phl p 5) to determine his allergogram (Laffer et al., 1994; Doleck et al., 1993; Vrtala et al., 1993a).

Preparation of RNA and PCR Amplification of cDNAs Coding for IgE Heavy Chain Fragments and Light Chains from Peripheral Blood Mononuclear Cells of the Grass Pollen Allergic Patient—150 ml of heparinized blood were obtained from the allergic donor during the grass pollen season (with informed consent). Peripheral blood mononuclear cells were prepared by Ficoll-Paque density gradient centrifugation (Pharmacia) (Steinberger et al., 1995). RNA was prepared by a guanidinium isothiocyanate method (Davis et al., 1986). Several independent cDNA synthesis and PCR amplification reactions were carried out using a RNA PCR kit (Perkin-Elmer). In brief, total RNA (20–60 µg) was mixed with 10–20 pmol of oligonucleotide primers specific for the constant region of the epsilon chains (C10968 et al., 1991) and mimic myoglobin were amplified with the RIACT kit (Pharmacia, Uppsala, Sweden) to be 5% of total DNA. The wells were blocked with phosphate-buffered saline containing 3% (w/v) bovine serum albumin. Freshly prepared phage suspension (approximately 10^2 plaque-forming units) was added to each well and incubated at room temperature for 2 h. The phage were then removed, and the wells were washed with Tris-buffered saline containing 0.05% (w/v) Tween 20 once. Phage were eluted with 0.1 M glycine-HCl, pH 2.2, containing 1 mg/ml bovine serum albumin, and the eluent was neutralized with 2× Tris. Freshly grown bacteria were used for the infection. The supernatant produced from the bacterial ELISA plating test incubated with the eluted phage was used to determine the titer of infected cells. The culture was grown in SB medium containing 50 µg/ml ampicillin and 10 µg/ml tetracyclin. By infection with helper phage VCS M13, filamentous phage were produced for the next round of panning as described (Barbas et al., 1991). The titer of infected cells was determined four times. During the subsequent pannings, additional washing of the wells was done and individual clones were then analyzed for the production of Phl p 5-specific Fab by ELISA.

Sequence Analysis of the cDNAs Coding for IgE Fds and Light Chains—Clones 5, 14, 28, and 31 were checked for the production of Phl p 5-specific Fab by ELISA and for the correct insertion of cDNAs coding for heavy chain fragments and light chains by restriction analysis before sequencing. Plasmid DNA was prepared from recombinant E. coli XL-1 Blue using Qiagen tips (Hilden, Germany). Both DNA strands were sequenced using 33S-dCTP (DuPont NEN) and a T7 polynuclease sequencing kit (Pharmacia) by primer walking (Sanger et al., 1977). Sequencing primers were obtained from Pharmacia. The DNA amplicons were released from the head of the T7 polymerase. The heavy and light chains were compared with the GenBank and Swissprot library.

Production of Soluble Recombinant Fab Fragments with Specificity for Phl p 5—For the production of soluble Fab fragments, DNA was isolated from several independent clones after the fifth round of panning. The plasmid DNA was digested with SpeI and HinfI, recovered from a 3% agarose gel, self-ligated, and retransformed into E. coli XL-1 Blue. E. coli containing the correctly religated plasmid were then used to produce soluble Fab fragments. In brief, single colonies were inoculated into SB medium containing 20 µg/ml M9 and 50 µg/ml carbencillin. The cultures were grown at 37 °C for 6 h and then induced by adding isopropyl-thio-b-galactopyranoside to a final concentration of 4 µM. Induced E. coli were then grown at 30 °C overnight, and cells were harvested by centrifugation at 3000 × g for 10 min at 4 °C. The E. coli supernatants were used for ELISA assays, immunoblotting, and for the affinity purification of Phl p 5-specific Fab.

Purification of Phl p 5-specific IgE Fabs by Affinity Purification—Recombinant Phl p 5-specific Fab was coupled to an AminoLink™ column (Pierce) according to the manufacturer's advice. Approximately 200 ml of E. coli supernatant containing Phl p 5-specific Fab were centrifuged at 20,000 × g and subsequently filtered through folded filters (Machery-Nagel, Düren, Germany) to remove debris from the solution. The supernatants were applied to the column at 4 °C, and the column was washed extensively with phosphate-buffered saline until no protein could be detected by photometry at 280 nm in the wash fractions. Bound IgE Fab was eluted from the column with 0.1 M glycine-HCl, pH 2.2, containing 1 mg/ml bovine serum albumin, neutralized in 3M Tris, pH 9.
were confirmed to represent IgE-Fd encoding fragments by a high proportion of allergen-specific IgE-Fds. The PCR products obtained were analyzed by electrophoretic mobility to determine the size of the amplified products. The position of group 1 and group 5 allergens was determined by SDS-PAGE and subsequent staining. The PCR products were purified and sequenced to determine the amino acid sequence of the IgE-Fds.

Since the patient did not receive hyposensitization therapy, it is not surprising that the efficacy of the IgE combinatorial library was not observed. However, it is noteworthy that the increased IgE production during the grass pollen season was mostly due to stimulation by allergen extracts from rye grass, Kentucky Bluegrass, rye, and timothy grass. Most of the allergens were detected by ELISA for the presence of IgE-Fds and light chains.

PCR Amplification of cDNAs Coding for IgE Fds and Light Chains from Peripheral Blood Lymphocytes of a Grass Pollen Allergic Individual—Starting from RNA of peripheral blood lymphocytes of a grass pollen allergic patient, cDNAs coding for IgE-Fds could be amplified. Fig. 2 shows the successful PCR amplification of IgE-Fds using primers specific for different VH-gene families. RNA was isolated at different times of the grass pollen season and is noteworthy that the efficacy of the PCR amplification was best during the grass pollen season.

The obtained PCR product therefore may contain a high proportion of allergen-specific IgE-Fds. The PCR products were confirmed to represent IgE-Fd encoding fragments by differential hybridization using synthetic oligonucleotides specific for IgE and IgG as described (Steinberger et al., 1995).

Construction and Characterization of an IgE Combinatorial Library from a Grass Pollen Allergic Patient—Subcloning of light chain cDNAs into plasmid pComb3H yielded a plasmid library consisting of 3 × 10⁷ primary clones. The subsequent ligation of the IgE-Fd cDNAs led to the construction of a combinatorial library of approximately 5 × 10⁷ primary clones which had contained heavy and light chain cDNAs. Fig. 3 shows the restriction analysis of 20 randomly isolated clones of the IgE combinatorial library. Only seven clones did not contain both cDNAs coding for the heavy chain fragment and light chain, indicating that 65% of the clones had combined correctly both cDNAs.

Isolation and Characterization of Recombinant Human IgE Fabs with Specificity for the Major Timothy Grass Pollen Allergen Phl p 5—Filamentous phage expressing IgE Fabs on their surface were screened using ELISA for the presence of Phl p 5-specific Fabs, among which four Phl p 5-specific clones designated clone 5, 14, 28, and 31 were characterized in detail. The specificity of the supernatants for Phl p 5 was confirmed by testing them for binding to recombinant Phl p 5.

Both strands of the DNA sequences coding for the Fd fragments and light chains of the clones were determined according to Sanger by primer walking, and the amino acid sequence was deduced. Fig. 4 shows the DNA and deduced amino acid sequences of the IgE heavy chain fragment, which was utilized by all four clones. It is noteworthy that identical heavy chain fragments were obtained by using two different PCR primers for the C H domain, indicating a positive selection for these particular IgE Fds during the panning process. The parts of the C H domain were completely identical with known human IgE sequences (Flanagan and Rabbitts, 1982; Kurakawa et al., 1983). A molecular mass of 24.2 kDa could be predicted for the Fd-fragments of clones 5 and 28, whereas a molecular mass of 22.5 kDa could be deduced for clones 14 and 31, which were generated by a constant region primer located closer to the variable region (Fig. 4).

As can be seen in Fig. 5 (A and B), different κ light chains
were used by the four clones. Most of the differences in the nucleotide sequences of the framework regions were silent. Regarding the CDRs of the four light chains, most differences were found in the CDR3 and CDR1. In conclusion, the panning procedure with recombinant Phl p 5 had enriched different IgE Fabs, which used identical heavy chain fragments that had combined with different light chains.

Recombinant Human IgE Fabs Specific for Phl p 5 Cross-react with Group 5 Allergens from Three Different Grass Species—More than 80% of grass pollen allergic patients display IgE cross-reactivity to group 5 allergens (Vrtala et al., 1993a). cDNAs coding for functional recombinant group 5 allergens were isolated from these species (Singh et al., 1991; Silvanovic et al., 1991; Vrtala et al., 1993a) and shown to be highly homologous. In order to investigate whether the recombinant human IgE Fabs that were isolated by panning to recombinant Phl p 5 cross-react with natural group 5 allergens from different grass species, grass pollen extracts from rye grass, Kentucky Bluegrass, rye, and timothy grass were probed. Fig. 6 shows that supernatants containing soluble Fabs from clones 5 and 28 reacted with nitrocellulose blotted rye grass (L. perenne), Kentucky Bluegrass (P. pratensis), rye (S. cereale), and timothy grass (P. pratense) pollen extract at approximately 30 kDa, which corresponds to the molecular mass of group 5 allergens. Additional weak binding to a 17–20 kDa component was observed in Kentucky Bluegrass and rye. Group 6 allergens, which share a high degree of sequence homology with group 5 allergens, were described to migrate at that molecular mass; however, the question whether group 6 allergens represent cleavage products of group 5 allergens or cross-reactive allergens is not yet clear (Matthiesen et al., 1993).

No reactivity of the Phl p 5-specific IgE Fabs was observed with birch pollen extract, which does not contain group 5 allergens. A recombinant IgG Fab (Co) with specificity for the major birch pollen allergen Bet v 1 was included as control and showed no reactivity with grass pollen extracts, whereas it bound to Bet v 1 at 17 kDa in birch pollen extract.

Recombinant human IgE Fabs, which were isolated by panning to purified recombinant Phl p 5, cross-reacted with natural group 5 allergens from different grass species as is known for natural IgE antibodies from grass pollen allergic patients.

Purification of Soluble Recombinant IgE Fabs Specific for...
blotted recombinant Phl p 5 up to dilutions of 1:500000 (data not shown). A single purification procedure starting from about 200 ml E. coli supernatant yielded approximately 0.5 mg of pure and soluble recombinant Fab.

Recombinant IgE Fabs Specific for Phl p 5 Compete with Allergic Patients IgE Binding—Using competition experiments, it was investigated whether recombinant Phl p 5-specific IgE Fabs might be able to compete with grass pollen allergen-specific IgE antibodies. Pairs of nitrocellulose strips containing equal amounts of blotted recombinant Phl p 5 were preincubated with supernatants from two Phl p 5-specific Fab clones (Fig. 8, lanes 5 and 31) or with supernatants from E. coli expressing Bet v 1-specific Fab clones (Fig. 8, Co). The nitrocellulose strips were then incubated with serum IgE from two patients (A and B), and bound IgE was detected. Preincubation of Phl p 5 with the Phl p 5-specific IgE Fabs led to a weak but clearly visible reduction of IgE binding compared to the control. The weak competition was not unexpected in view of the fact that Phl p 5 harbors multiple IgE epitopes (Bufe et al., 1994).

DISCUSSION

The cross-linking of effector cell-bound IgE antibodies by allergens has been recognized as the key event leading to Type I allergic reactions. Although IgE antibodies are present at extremely low levels in serum (10–400 ng/ml), the release of mediators triggered by the cross-linking event causes severe allergic reactions (rhinitis, conjunctivitis, allergic asthma, and anaphylaxis). For this reason immunoglobulin E has been characterized extensively by protein chemical and molecular biological techniques (Terry et al., 1970; Kochwa et al., 1971; Bennich et al., 1973; Flanagan and Rabbits, 1982; Kurokawa et al., 1983; Seno et al., 1983). Whereas considerable progress was achieved regarding the characterization of the constant regions of IgE, in particular the binding site for the high affinity receptor (Helm et al., 1988; Nissim and Esnahr, 1992; Presta et al., 1994), nothing was known about the V regions of IgE antibodies with specificities for allergens. In the present study we have used the filamentous phage display system to isolate human allergen-specific recombinant IgE Fab fragments. To achieve this goal, a highly sensitive PCR technique was established to allow the amplification of IgE Fd from the peripheral blood of allergic patients (Steinberger et al., 1995). An IgE combinatorial library was constructed in the pComb3H plasmid, starting from peripheral blood lymphocytes from a grass pollen allergic patient. Using purified recombinant timothy grass pollen allergens for the panning procedure, human IgE Fabs with specificity for the major timothy grass pollen allergen Phl p 5 could be isolated. Phl p 5 was used as a model allergen, because it represents a major allergen for more than 80% of grass pollen allergic individuals and cross-reacts with group 5 allergens from most grass species (van Ree et al., 1992). Another reason for selecting Phl p 5 was the fact that a high percentage of grass pollen-specific IgE antibodies are directed against this allergen in most patients (Vrtala et al., 1993a, 1994). Serum from the patient who was used for the construction of the combinatorial library contained high levels of Phl p 5-specific IgE compared to Phl p 1-specific IgE (Fig. 1). In fact, many more phage clones with specificity for Phl p 5 could be recovered from the combinatorial library after five rounds of panning than clones that reacted with Phl p 1.

It appeared hence that the repertoire represented in the IgE combinatorial library closely reflected the natural IgE antibody repertoire.

2 P. Steinberger, D. Kraft, and R. Valenta, unpublished data.
Characterization of Allergen-specific Human IgE Fabs

The sequence analysis of four independent Phl p 5-specific IgE Fabs revealed that all four clones used the same type of heavy chain fragment originating from different PCR reactions, which had recombinated with different kappa light chains. The finding that different PCR products of the same IgE Fd and similar light chains had combined to form the Fabs that were selected by the panning procedure indicated that the recombinant Fabs most likely were produced in response to recombinatorial library most likely were produced in response to repeated allergen stimulation.

Like the natural IgE antibodies, the Phl p 5-specific IgE Fabs cross-reacted with group 5 allergens from rye grass, Kentucky Bluegrass, and rye. Using immobilized recombinant Phl p 5, soluble human IgE Fabs could be purified to homogeneity up to milligram amounts. Due to a lack of the cc2-cc4 domain, the recombinant IgE Fabs were ineffective to trigger basophil degranulation in combination with purified Phl p 5 (data not shown). In addition it could be shown that the IgE Fabs were able to compete with the IgE binding to Phl p 5 using sera from grass pollen allergic individuals. The inhibitory effect was, however, very weak, which was not surprising in view of the fact that Phl p 5 bears a number of different IgE epitopes (Burke et al., 1994).

The finding that different PCR products of the same IgE Fd and similar light chains had combined to form the Fabs that were selected by the panning procedure indicated that the recombinant Fabs most likely were produced in response to repeated allergen stimulation.

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