

Cloning of cDNA Coding for an Allergen of Cocksfoot Grass (*Dactylis glomerata*) Pollen

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Abstract. Messenger RNA isolated from Cocksfoot grass (*Dactylis glomerata*) anthers has been used to generate a cDNA library in λ t11. Three cDNA clones (7.8, 8.1, and 8.3) were demonstrated to be recognized by human IgE antibodies in atopic serum and by rabbit polyclonal antiserum raised to a crude aqueous extract of Cocksfoot pollen. The size of the cDNA inserts was determined as approximately 700 bp, and restriction mapping demonstrated them to be identical sequences. Lysogens obtained in *Escherichia coli* Y1089 allowed expression of a 140 kD β -galactosidase fusion protein containing 24 kD of cloned allergen protein. Fusion proteins were recognized by IgE antibodies in 75% (6/8) of atopic sera tested, but were not detected by non-atopic sera. On the basis of size and frequency of recognition in the atopic population, the cloned protein may represent a major allergen. Monoclonal antibodies specific for the major allergen of Cocksfoot pollen were not reactive with the fusion proteins. Reactivity of human IgE antibodies with the fusion protein could be blocked by crude Cocksfoot pollen extract, but not by the major allergen DG3 purified from the extract by affinity chromatography. Human and rabbit antibodies affinity purified against fusion protein 7.8 did not allow identification of the native protein component in crude extract encoded for by the cDNA clones.

Introduction

The pollen of Orchard or Cocksfoot grass (*Dactylis glomerata*) is a significant causative agent of allergic rhinitis (hayfever) in temperate regions [1-3]. Aqueous extracts of this and other pollens used in immunotherapy are frequently complex, ill-defined mixtures of antigenic components, of which only a few are significant for therapy, and some are potentially toxic, thereby interfering with both diagnosis and treatment [4]. Some highly purified allergens isolated from a variety of pollens by immunochemical and physicochemical separations [5-9] have been shown to be superior to crude extracts in immunotherapy [10].

Purified reagents have also facilitated the study of allergen immunochemistry and the genetic basis of human immune responsiveness in allergic individuals [11, 12]. Currently, however, there have been few reports of allergens isolated by recombinant DNA technology. Cloning and sequencing of allergen cDNAs is a potentially faster method of determining their ami-

no acid sequence compared to direct sequencing of the proteins themselves. Establishment of allergen encoding cDNA clones also has additional advantages. Expression of the clones as proteins will enable high purity, defined allergens to be produced in bulk in vitro for use as desensitizing agents in vivo. Site-directed mutagenesis of allergen cDNA clones may provide information on size, structure, and immunogenicity of allergens. This will enable the epitopes recognized by human IgE and IgG antibodies to be mapped and allow the construction of cDNA clones encoding allergens that are modified to allow induction of IgG antibodies, but not IgE antibodies. Since it is the IgE-allergen complexes which mediate histamine release via interaction with the high-affinity human IgE receptor (Fc ϵ RI) [13] on human mast cells and basophils whilst it is believed that IgG antibodies block allergic sequelae [14, 15], such allergens may avoid induction of allergic responses without inhibiting the production of protective IgG antibodies. In this paper we describe the cloning of a cDNA encoding an allergen from the pollen of Cocksfoot grass.

Materials and Methods

Bacterial and Phage Strains

E. coli strains Y1089 and Y1090 and lambda gt11 have previously been described by Young and Davis [16].

Isolation of RNA

Total RNA was isolated from grassheads harvested from wild plants between 10 and 2 days before anthesis. Tissue was ground in liquid nitrogen, then in 1 vol extraction buffer: 0.5 vol phenol/cresol and homogenized with a polytron homogenizer. Extraction buffer was 400 mM NaCl, 50 mM Tris (pH 9), 50 mM EDTA (pH 8), 1% sodium dodecyl sulphate, 10 mM dithiothreitol, 4 U/ml heparin, and 100 mM aurintricarboxylic acid. Phenol/cresol contained 40% (w/v) phenol, 0.04% (w/v) 8-hydroxyquinoline, and 12% (v/v) cresol in 50% (v/v) extraction buffer. The extract was clarified by centrifugation, the supernatant extracted with phenol/chloroform (1:1 v/v), and RNA precipitated with 2 M LiCl (final concentration). Poly (A)⁺ RNA was prepared by oligo (dT) cellulose chromatography as described by Maniatis et al. [17].

Antisera

Antibodies against Cocksfoot grass pollen were raised by repeated subcutaneous injection of rabbits with 0.5 mg of an aqueous crude extract [18] of pollen emulsified in an equal volume of Freund's complete adjuvant every 3–6 months. Serum from the best-responding rabbit gave a precipitin line against crude pollen extract using Ouchterlony's double-diffusion analysis with a titre of 1:16 dilution.

Human serum samples were obtained from the Clinical Chemistry Department, Queen Elizabeth Hospital, Birmingham, and were assayed for Cocksfoot grass pollen specific IgE antibodies by enhanced chemiluminescent immunoassay [19]. Equal volumes of sera giving high or low values in the assay were pooled to constitute an atopic and non-atopic serum pool, respectively.

Construction and Screening of cDNA Libraries

Double-stranded cDNA was prepared from poly (A)⁺ RNA as described by Watson and Jackson [20], except that unligated linkers and small cDNA fragments were separated from larger products by high-performance liquid chromatography [21]. cDNA was inserted into the Eco RI site of λ t11 as described by Huynh et al. [22] and the DNA packaged using a Gigapack Plus packaging extract according to the supplier's instructions (Stratagene Cloning Systems, San Diego, Calif., USA).

Libraries were immunoscreened according to the Promega Protoblot[®] manual using a 1:1,000 dilution of rabbit pollen specific antiserum preabsorbed with *E. coli* lysate or a 1:20 dilution of human atopic serum pool. Alkaline phosphatase conjugated second antibodies (swine anti-rabbit IgG and sheep antihuman IgE; Dako-patts) were used at 1:1,000 dilution in phosphate-buffered saline. Detection of bound second antibody was performed using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate system [23]. Phages from immunopositive plaques were amplified by the plate lysate method [17].

Preparation of Recombinant Antigen

Lysogens were obtained in *E. coli* Y1089 as described [22] using immunopositive λ t11 clones and grown in liquid culture to OD₆₀₀ = 0.4 at 32°C. Cultures were incubated at 42°C for 20 min and synthe-

sis of fusion proteins induced by addition of 10 mM isopropyl B-D thiogalactoside (IPTG). After 1 h at 37°C, the cultures were harvested as described by Gupta et al. [24].

Western Blotting

Lysogen extracts were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) in 8.5% cross-linked gels [25]. After electrophoresis, proteins were electrotransferred to nitrocellulose at 250 mA for 2 h using a BioRad Trans-blot[®] apparatus according to the manufacturer's recommendations. Blots were blocked by incubation with 2% bovine serum albumin in Tris-buffered saline for 30 min, incubated with first antibody overnight at 4°C and second antibody for 1 h at room temperature (each diluted in 1% bovine serum albumin in Tris-buffered saline), and revealed as described in the Promega Protoblot manual.

Analysis of cDNA Inserts

DNA was prepared from λ t11 clones by the miniprep method [26]. Amplification of insert sequence in recombinant clones was achieved using the polymerase chain reaction [27]. Extension of primers specific for β -galactosidase sequences flanking the insert (see legend to figure 3) was performed using Taq DNA polymerase (Anglian Biotech) according to a modification of the method of Stofflet et al. [28]. Restriction endonuclease digestion was performed on miniprep DNA with the enzyme Mlu I and on amplified products of the polymerase chain reaction with several enzymes including Xho I and Pvu I.

Results

Isolation of Pollen Allergen cDNA Clones

To investigate whether the λ t11 library contained clones expressing pollen protein epitopes, it was initially screened with rabbit polyclonal antiserum specific for proteins present in a crude aqueous extract of Cocksfoot pollen. Screening of a total of 3×10^4 plaques yielded nine immunopositive clones. These clones were amplified by the plate lysate method, and aliquots of each stock were spotted onto a lawn of *E. coli* Y1090 and rescreened with the human atopic serum pool. Clone 7.8 was found to express protein epitope(s) recognized by human IgE antibodies present in the atopic serum pool. Screening of a further 2×10^4 plaques with the atopic serum pool identified two additional immunopositive clones denoted 8.1 and 8.3. Rescreening of clones 7.8, 8.1, and 8.3 with the human non-atopic serum pool gave signal intensities equivalent to that given by a non-recombinant clone (fig. 1). None of the clones gave higher signals than a non-recombinant following screening with the atopic serum pool followed by detection using sheep antihuman IgG second antibody (data not shown).

The insert size in the clones 7.8, 8.1, and 8.3 was determined by digestion of miniprep lambda DNA with

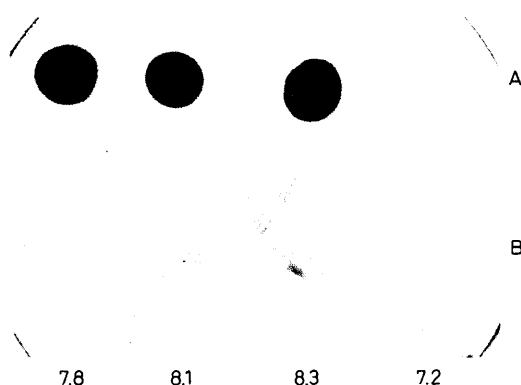


Fig. 1. Dot screening of purified gt11 clones on *E. coli* Y1090. Five microliters of phage stocks amplified from immunopositive clones 7.8, 8.1, and 8.3 and a non-recombinant, clone 7.2, were spotted onto a lawn of *E. coli* Y1090 induced with a nitrocellulose filter saturated with 10 mM IPTG and probed with (A) atopic and (B) non-atopic human serum pools.

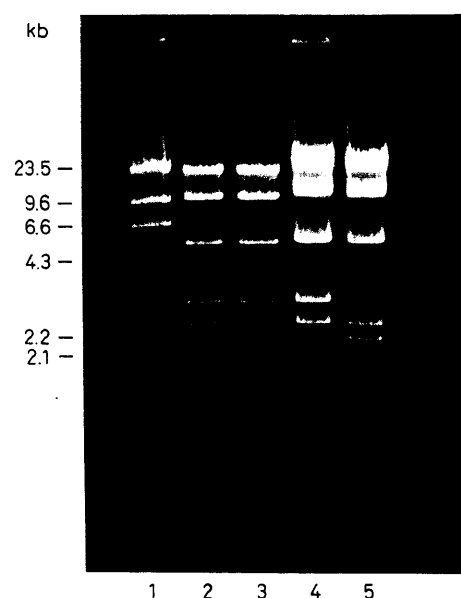


Fig. 2. Agarose gel separation of lambda miniprep DNA digested with the enzyme Mlu I. Lane 1: Hind III size markers; lane 2: clone 7.8; lane 3: clone 8.1; lane 4: clone 8.3; lane 5: a non-recombinant clone.

Mlu I followed by electrophoretic separation of fragments in a 0.8% agarose gel (fig. 2). The non-recombinant 2-kilobase (kb) fragment contains the Eco RI cloning site and is thus of variable size in the λ t11 recombinants. Each clone (7.8, 8.1, and 8.3) gave bands equivalent to inserts of approximately 700 base pairs (bp). Amplification of the insert region using the polymerase chain reaction followed by agarose gel electrophoresis revealed fragments of approximately 720 bp for each clone.

Restriction enzyme analysis of the amplified polymerase chain reaction products revealed unique sites for Pvu I and Xho I at identical positions in the inserts for each clone, demonstrating them to be identical (fig. 3).

Immunological Identification of Recombinant Proteins

Confirmation of the immunopositive recombinants as pollen allergen clones was provided by analysis of fusion proteins in lambda lysogens on western blots following electrophoresis of lysates in SDS-PAGE (fig. 4, 5).

A fusion protein of approximately 140 kD was identified in lysates from induced lysogens (fig. 4, lanes 3, 5, 7) using anti- β -galactosidase antibody but not in non-induced lysogens (lanes 2, 4, 6). A λ t11

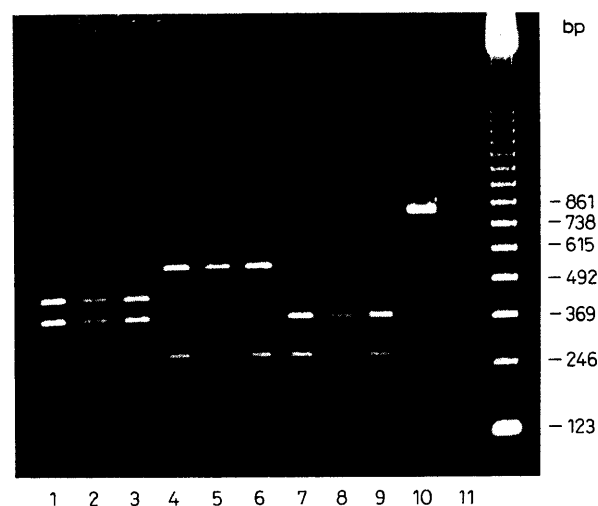


Fig. 3. Agarose gel separation of polymerase chain reaction amplified inserts from clones 7.8 (lanes 1, 4, 7), 8.1 (lanes 2, 5, 8), and 8.3 (lanes 3, 6, 9) digested with the enzyme Pvu I (lanes 1-3), Xho I (lanes 4-6), and Pvu I + Xho I (lanes 7-9). Lane 10 contains an undigested insert from clone 7.8 and track 11 an undigested insert from a non-recombinant clone. Forward primer: 5'd (GACTCCTCGAGCCCCG)3'. Reverse primer: 5'd(TTGACAC-CAGACCAACTGGTAATG)3'.

Fig. 4. Immunological identification of β -galactosidase and pollen allergen epitopes in the fusion proteins on western blots taken from 8.5% cross-linked SDS-PAGE gels. Lane 1: induced λ t11 lysogen from nonrecombinant clone; lanes 2 and 8: non-induced 7.8 lysogen; lanes 3, 9, and 12: induced 7.8 lysogen; lane 4: non-induced 8.1 lysogen; lanes 5, 10, and 13: induced 8.1 lysogen; lane 6: non-induced 8.3 lysogen; lanes 7, 11, and 14: induced 8.3 lysogen. Lanes 1-7 were incubated with anti- β -galactosidase antibody, 8-11 with human atopic serum, and 12-14 with non-atopic serum and revealed using alkaline phosphatase conjugated antimouse IgG and antihuman IgE second antibodies, respectively. Molecular weight markers were myosin (200 kD), β -galactosidase (116.3 kD), phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), and ovalbumin (42.7 kD).

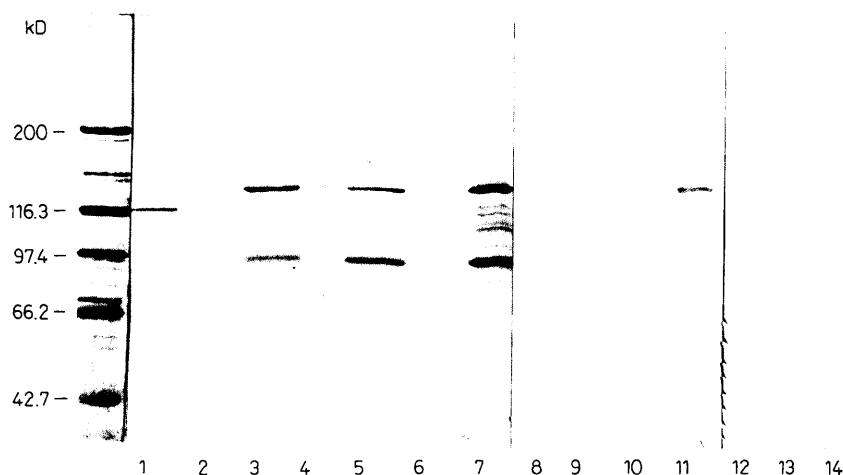
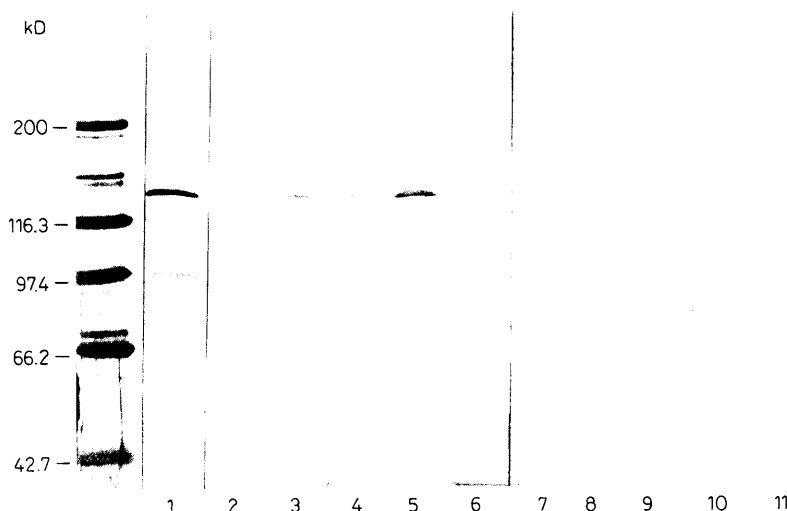


Fig. 5. Immunological identification of pollen allergen epitopes recognized by rabbit or human antibodies purified by plaque antibody selection using clone 7.8, following western blotting of an 8.5% cross-linked SDS-PAGE gel. Lanes 1, 3 and 9: induced 7.8 lysogen extract; lanes 2 and 8: non-induced 7.8 lysogen; lanes 4 and 10: induced 8.1 lysogen; lanes 5 and 11: induced 8.3 lysogen; lanes 6 and 7: induced λ t11 non-recombinant lysogen. Lane 1 was incubated with anti- β -galactosidase antibody, lanes 2-6 with rabbit antibodies purified on clone 7.8, lanes 7-11 with human antibodies purified on clone 7.8 and revealed using alkaline phosphatase conjugated antimouse IgG, antirabbit Ig and antihuman IgE second antibodies, respectively.



lysogen without any cDNA insert showed an induced β -galactosidase protein which comigrated with the 116-kD β -galactosidase marker (lane 1).

Fusion proteins in induced lysogen lysates from all three clones were reactive with IgE antibodies in the human atopic serum pool (fig. 4, lanes 9, 10, 11), but were not detected following incubation with human non-atopic serum pool (fig. 4, lanes 12-14). Polypeptides in the induced lysates detected by the anti- β -galactosidase antibody ranged from 116 to 66 kD with a major band at approximately 95 kD, but these were not detected by human IgE antibodies in the atopic serum pool, suggesting that they represent breakdown

products of the fusion protein which do not contain the allergenic epitope(s). A blot (fig. 5) probed with rabbit and human antibodies positively affinity purified using immobilized fusion protein from clone 7.8 (FP 7.8) revealed a protein that comigrated upon electrophoresis with the fusion protein revealed with anti- β -galactosidase antibody (fig. 5, lane 1). This protein band was only detected in lysates of induced recombinants (lanes 3-5, 9-11) and not in lysates of non-induced recombinants (lanes 2, 8) or induced non-recombinants (lanes 6, 7). Thus, rabbit and human antibodies purified using FP 7.8 also reacted with fusion proteins from clones 8.1 (FP 8.1) and 8.3 (FP 8.3).

FP 8.3 was probed on a western blot with eight atopic sera and four non-atopic sera and revealed with alkaline phosphatase conjugated sheep antihuman IgE. Specific IgE was detected in 6 of 8 (75%) and 0 of 4 (0%) of the atopic and non-atopic sera, respectively (data not shown). None of the fusion proteins were recognized by murine monoclonal antibodies generated to the major allergenic protein of Cocksfoot grass DG3 which may be identical to Dac g I [manuscript in preparation].

Inhibition of the reaction of rabbit polyclonal antibodies and human IgE antibodies in the atopic serum pool with the fusion proteins on western blots was evident using an aqueous crude extract of Cocksfoot pollen; however, DG3 immunopurified from the crude extract was unable to inhibit at concentrations tenfold greater than that present in the crude extract (data not shown).

Discussion

In this report we describe the cloning of a cDNA sequence encoding an allergen from the pollen of Cocksfoot grass. Several lines of evidence indicate that the clone contains pollen allergen sequence. Firstly, the clone was immunoselected from the λ t11 expression library with a human serum pool containing high levels of Cocksfoot grass pollen specific IgE antibodies, but was undetected using serum with low specific IgE levels. Secondly, the protein product of the cDNA sequence, expressed as a fusion protein with β -galactosidase, was identified on a western blot with atopic but not non-atopic serum. Rabbit polyclonal antiserum specific for proteins in the crude extract of Cocksfoot pollen also recognized the fusion protein. However, human IgE antibodies purified from atopic serum using the fusion protein FP 7.8 did not react with components in electrophoresed crude Cocksfoot pollen extract, thus preventing identification of the native protein. The fact that 75% of atopic sera tested contained IgE antibodies reactive with the fusion protein suggests that the clone encodes a protein contributing to the allergic situation in vivo in a large portion of the atopic population and could represent a 'major' allergen.

On the basis of size determined by miniprep and polymerase chain reaction amplification of the insert and restriction enzyme mapping, we conclude that the clones 7.8, 8.1, and 8.3 contain identical inserts of ap-

proximately 700 bp. This has the capacity to code for a protein of approximately 25 kD, and probing of fusion proteins in induced lysates by anti- β -galactosidase and atopic serum antibodies gave a value of approximately 24 kD. Whether this is the full sequence of the allergenic protein remains to be determined, since human IgE antibodies purified using FP 7.8 were unreactive with proteins in the crude aqueous extract of Cocksfoot pollen. The identity of the native protein counterpart of these clones in the crude extract remains to be established, and it is anticipated that this will be achieved by raising polyclonal antisera to the fusion proteins.

We have isolated a major allergen from Cocksfoot pollen which we termed DG3 [18] which has similarities to Dac g I [29, 30], but we have been unable to establish identity due to lack of available sequence data or Dac g I specific antibodies. DG3 has, however, been demonstrated to be reactive with IgE antibodies in >95% of atopic sera [manuscript in preparation]. DG3 was unable to inhibit binding of human IgE or rabbit polyclonal allergen specific antibodies to the fusion proteins, whereas the crude extract did. Similarly, DG3-specific murine monoclonal antibodies did not recognize the fusion protein. Therefore, we conclude that these clones are not expected to code for the major allergen of Cocksfoot grass pollen termed DG3 which may be different to Dac g I. The demonstration that the fusion protein is recognized by IgE antibodies in 75% of atopic sera tested suggests that they encode proteins which may be major rather than minor allergens.

We are currently attempting to generate amino acid sequence data on both DG3 and the fusion proteins in order to generate oligonucleotide probes to rescreen the cDNA library. The DNA sequence of the clones will also be established using a modification of the polymerase chain reaction and by raising antisera to the fusion proteins. We hope to establish the identity of the native protein counterparts in Cocksfoot pollen. Armed with these data we may be able to perform site directed mutagenesis in order to define the structure of the epitope(s) recognized by human IgE and IgG antibodies. This may allow the generation of purified mutated forms of the allergens that can elicit a predominant IgG response, thereby blocking the allergic sequelae without impairing generation of the protective immune responses, and which would, therefore, be applicable for use as desensitizing agents in vivo.

Acknowledgements

The authors would like to thank Philip Day for help with the polymerase chain reaction and Dr. Chris Franklin for assisting with the cloning. This work was supported by a grant from the Medical Research Council, UK, and by the Department of Health, UK.

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Received: January 2, 1989

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