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A Species-Specific Monoclonal Antibody to *Cynodon dactylon*

Abstract

Background: It is usually difficult to differentiate between the pollens of different grass species on the basis of their appearance under a microscope, as they often appear similar. Such distinctions are important when interpreting the clinical relevance of pollens in air samples as individuals can differ in their allergic responses to different grass species. As this allergenic distinction occurs at the level of presence and differences of epitopes on the allergens associated with different species, it could be anticipated that species-specific monoclonal antibodies could provide such distinctions between pollens. **Method:** Monoclonal antibodies raised against *Cynodon dactylon* were screened and characterised in ELISA assays and blotting, using a range of grass pollen extracts, to identify clones which were species specific. **Results:** The most specific monoclonal raised to *C. dactylon* did not react at a level of greater than 1.2% to extracts of 10 other grass pollens in a direct ELISA assay and showed no detectable cross-reactivity in a particle blotting assay. **Conclusion:** It has been possible to produce a monoclonal antibody that is functionally species specific to *C. dactylon*.

Key Words

Grass pollen
Monoclonal antibodies
Species specificity

Introduction

Grass pollen allergies are common in many regions of the world. Bermuda grass (*Cynodon dactylon*) and Ryegrass (*Lolium perenne*) are among the most clinically important sources of grass pollen allergens. The level of allergenic distinction between their group I allergens (Cyn d 1 and Lol p 1, respectively) is unclear. Although Cyn d 1 and Lol p 1 have been well characterised [1–5] and share a high level of sequence homology, it has been suggested that Lol p 1 may possess an additional epitope not present in Cyn d 1 [6], though it is not known whether Cyn d 1 possess any unique, additional important epitopes. Previous studies using both monoclonal and rabbit polyclonal antibodies to *C. dactylon* have shown a high degree of cross-reactivity to other grasses [4, 6–9] and even to non-grasses [10–14]. We have therefore attempted to develop monoclonal antibodies

that would only bind to *C. dactylon* without any cross-reactivity, especially of the closely related pooidae grasses. Such species-specific antibodies could be used to give a very precise index of such exposure.

Material and Methods

Pollens

The following dry pollens were purchased from Greer Laboratories Inc. (Lenoir, N.C., USA): *Sorghum halepense* (Johnson grass), *Paspalum notatum* (Bahia grass), *C. dactylon* (Bermuda grass), *Phleum pratense* (Timothy grass), *Holcus lanatus* (Velvet grass), *Festuca elatior* (Fescue grass), *Dactylis glomerata* (Orchard grass), *Lolium perenne* (Rye grass), *Poa pratensis* (Kentucky Bluegrass), *Phalaris arundinaceae* (Canary grass, reed), *Anthoxanthum odoratum* (Sweet vernal grass). Pollen from the Birch tree (*Betula verrucosa/pendula*) was used as a control.

Extraction and Fractionation

One-gram lots of pollens were extracted in 10 ml of 0.1 M borate buffer pH 8.2 with phenyl methyl sulphonyl fluoride (Sigma P 7626) for 3 h at room temperature on a end-over-end mill. After centrifugation, the protein content of the extract was determined with a conventional protein assay (Bio-Rad No. 500-0006). Extracts were stored at -18°C .

The *C. dactylon* extract was initially fractionated by ion exchange chromatography (BioRad BioLogic) using a High Q Anion 10-ml bioscale column and 10 mM Tris, pH 8.8 and 150 mM Tris, pH 5.0 buffers. Ion exchange fractions were then analysed by SDS-PAGE (10% Novexgels and a Novex Xcell II) including electroblotting (Novex) to 0.45- μm nitrocellulose membranes and probing with IgE from a pool of sera from subjects allergic to grass pollens to identify fractions containing major allergens. *C. dactylon* fractions with purified proteins which could be bound by human IgE and that had a molecular weight corresponding to Cyn d 1 [6] were used to immunise mice to develop the monoclonal antibodies.

Development of Monoclonal Antibodies

BALB/c mice were immunised with three injections, each of 30 μg of the purified Cyn d 1 fractions. The first was in Freund's complete adjuvant, the second in Freund's incomplete adjuvant and then the third (booster) injection in phosphate-buffered saline. The number of immunisations was kept to a minimum to enable isolation of responses to the maximal number of epitopes, including potential non-shared epitopes.

The monoclonal antibodies were produced in cell culture, essentially using the method of Köhler and Milstein [15] (1975). Briefly, the NS1 cell line was used as fusion partner for spleen cells of immunised BALB/c mice. After fusion, the cells that were secreting *C. dactylon*-specific antibodies were cloned in limiting dilution three times for stabilisation. Isotype classification of antibodies was performed using a standard kit (Sigma ISO-2).

Immunocharacterisation of Antibody

ELISA Screening of Monoclonal Antibodies for Binding to Grass Pollen Extracts. ELISA wells were coated with 15 $\mu\text{g}/\text{ml}$ extracts of each of the 11 grass extracts (and the birch control extract) and then blocked with 5% skim milk protein. Five-fold serial dilutions of each monoclonal antibody (range 2 $\mu\text{g}/\text{ml}$ to 1 ng/ml) were incubated with each grass extract for 2 h, followed by washing, incubation with peroxidase-conjugated rabbit anti-mouse antibodies (Sigma A3673), development of colour with OPD/ H_2O_2 and reading in a microplate reader (BioRad model 550).

Particle Blotting. This is a novel assay system which enables the direct visualisation of antibody binding to allergens which have eluted from particles such as pollen grains [18]. Pollens from 11 different species of grasses and the birch control were separately sprinkled using a fine wire mesh sieve onto different areas of an adhesive film. This was then sandwiched with a protein-binding PVDF membrane (Millipore), the sandwich was briefly wetted in 50% ethanol and then incubated in phosphate-buffered saline for 3 h. The proteins eluting from the pollen grains bound to the membrane in the periphery of each grain. After blocking with 5% skim milk, the membrane-pollen-adhesive 'sandwiches' were incubated with monoclonal antibody number 3, specific for *C. dactylon*, and control antibodies specific for *L. perenne* and for the fungus *Alternaria alternata*. After washing, the

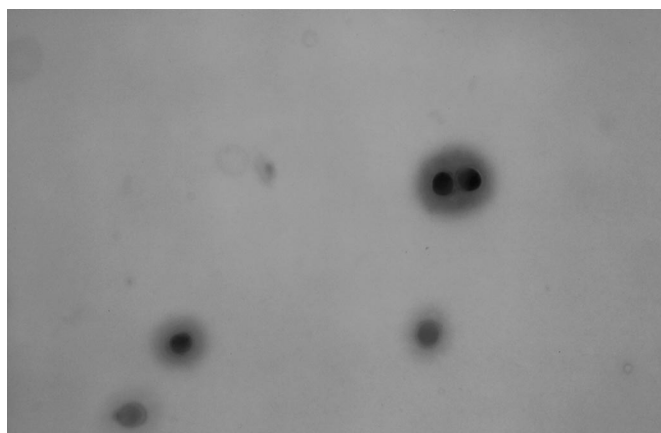


Fig. 1. Photos of immunologic recognition and halo formation around pollen particles. Mab 3 anti-*C. dactylon* using horseradish-peroxidase-conjugated secondary antibodies and DAB substrate develops a brown halo around pollen grains.

'sandwiches' were incubated with horseradish-peroxidase-conjugated anti-mouse antibodies (Sigma A3673) diluted 500 times. On incubation with the DAB substrate, a brown halo of staining is visible through the adhesive tape on the membrane associated with each pollen grain (fig. 1). This indicates binding between specific antibody and antigen eluted from the pollen grain.

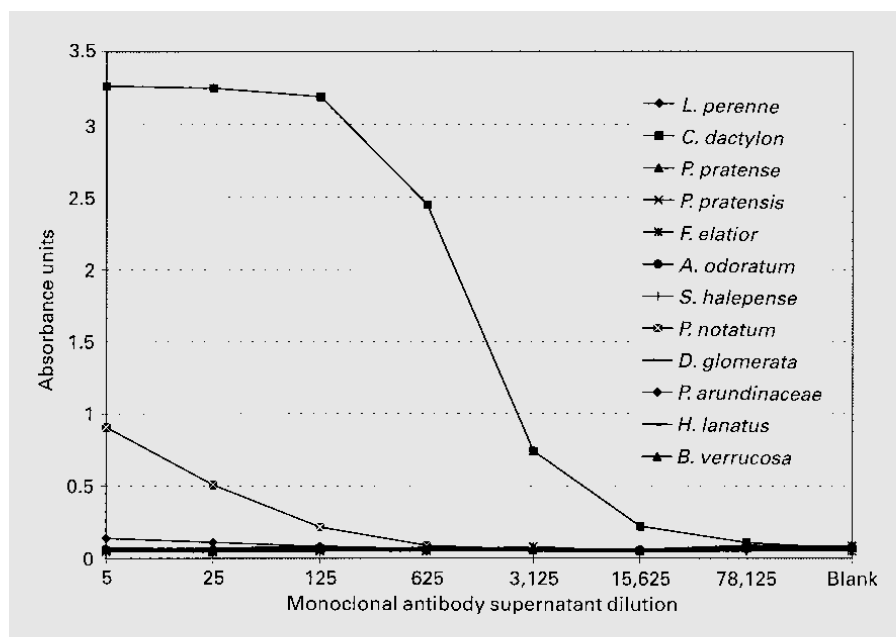
Immunoblotting. *C. dactylon* crude extract was electrophoretically separated on 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes as described previously. Blots were reacted with the selected monoclonal antibody (Mab3) or a non-cross-reacting control to determine the molecular weights of the components recognised. Monoclonal antibodies were incubated at 10 $\mu\text{g}/\text{ml}$ for 1 h, followed by horseradish-peroxidase-conjugated anti-mouse antibodies (Sigma A3673) for 1 h at 500 times dilution. Finally, DAB substrate gave band formation where Mab 3 had bound separated molecules.

Results

Pollen Extraction and Fractionation

Extraction of 1 g of *C. dactylon* pollen yielded 14.4 mg of soluble protein. This was the lowest protein yield for any of the pollens extracted. Ion exchange chromatography fractions, eluting at 14% buffer B (150 mM), contained a dense protein band of 31.2 kD molecular weight by SDS-PAGE, corresponding to the molecular weight of Cyn d 1. There was also some protein staining of the migration front and slight staining of higher molecular weight components. Blotting of these fraction with a grass-allergic serum pool showed strong binding of IgE to the 31.2-kD fraction.

Fig. 2. Mab 3 anti-*C. dactylon* grass reactivities. In the diagram each plot identifies the reactivity of one of the 11 tested grasses and control (*B. verrucosa*). Strong reactivity is seen to *C. dactylon* whereas the only other reaction (to *P. notatum*) is weak.



Development of Monoclonal Antibodies

On immunisation, all mice gave a strong antibody response which was detectable after 2 immunisations. After fusion, 5 cell lines, all IgG1 were stabilised and 1, called Mab 3, was selected as in the initial tests it consistently showed high specificity in direct ELISA towards *C. dactylon*, with very little or no cross-reactivity towards any other tested grass pollen extract. Antibody-secreting cells were grown to 0.5-litre scale.

Immunocharacterisation of Antibody Mab 3

ELISA Screening of Monoclonal Antibody Mab3 for Binding to Grass Pollen Extracts. The binding activity of Mab 3 to the 11 grasses plus control is shown in figure 2. There was strong binding to the *C. dactylon* extract. The binding to *P. notatum* was 1.2% of the binding to *C. dactylon* and there was no detectable response to any of the other 10 grasses and control, including *L. perenne*.

Particle Blotting. There was a strong response to *C. dactylon* on particle blotting, but no reaction to any of the other tested grass pollens or the control pollen (*B. verrucosa*). The negative control monoclonal antibody to *A. alternata* does not bind to any of the grasses, whereas the two other grass-specific monoclonal antibodies bind to 2 and 8 of the grasses (all Poaceae), respectively, showing narrow to broad activity. Figure 1 shows the microscopic view of particle blotting.

Immunoblotting. Mab 3 was tested in reaction towards crude *C. dactylon* extract. There was only reaction to one band corresponding to a molecule having a molecular weight of 31.1 kD. This band corresponded to a band previously recognised by human atopic sera as Cyn d 1.

Discussion

The development and characterisation of a monoclonal antibody which may be useful at a practical level for distinguishing and quantifying exposure to allergens from *C. dactylon* pollens, as distinct from other grass pollens, has been described. On the basis of molecular size characterisation and immunoassay studies, it would appear that the monoclonal antibody is specific for a non-IgE binding epitope on the Cyn d 1 allergen.

Single-step ion exchange chromatography was able to provide a sufficient purification of the major allergen fraction of the *C. dactylon* grass pollen extract, as determined by SDS-PAGE and protein-blotting with IgE. The purified fraction was highly immunogenic in mice, which showed a detectable immune response after 2 immunisations. Only 3 immunisations were performed in order to provide the widest diversity of epitopes and limit the opportunity of a few major epitopes dominating. From the resulting monoclonal antibodies, 1 was identified as particularly selective and is reported here.

The only pollen to show any detectable cross-reactivity in the ELISA assay was *P. notatum*, a member of the subfamily Panicoideae while *C. dactylon* is a member of the subfamily Eragrostoideae. No members of the subfamily Pooideae bound antibody Mab 3.

Mab 3 and human IgE show in immunoblotting binding to components with identical molecular weights, in parallel with this we find a positive response (data not shown) in monoclonal antibody-extract-IgE sandwich ELISA and no inhibition in ELISA testing IgE inhibition of Mab 3 binding to allergen, which indicates that Mab 3 and IgE bind through different epitopes on the Cyn d 1 allergen molecule.

The ELISA assay and the particle blotting assay gave slightly different results; in the ELISA there was strong binding by *C. dactylon* and weak binding (relatively about 1%) by *P. notatum* at high concentrations of antibody whereas in the particle blotting assay only the *C. dactylon* pollen showed binding. The difference is assumed to originate in the different ways of evaluation, where the ELISA

reader is more sensitive than the eye in microscopy. The antibody may however be used as discriminatory in ELISA also, if the dilution is 625 times or more, which is still giving a strong signal.

In conclusion we state that it has been possible to produce a monoclonal antibody to *C. dactylon* allergen that seems to be species specific, with only a trace response to 1 of the other 10 grasses tested. This monoclonal antibody may become important in assays of grass pollen allergens and allergies including type 1 allergy.

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