

Characterization of the major allergen of *Cynodon dactylon* (Bermuda grass) pollen, Cyn d I

Finn Matthiesen, MSc,* Michael J. Schumacher, MB, FRACP,** and
Henning Løwenstein, PhD, DrSc* Hørsholm, Denmark, and Tucson, Ariz.

An allergen from Cynodon dactylon (Bermuda grass) pollen, Cyn d I, has been purified by a combination of concanavalin A-Sepharose affinity chromatography, and carboxymethyl-Sepharose chromatography. The allergen constitutes the major allergenic component of the pollen extract as observed by immunoelectrophoretic techniques, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, an IgE-inhibition experiment, and skin testing. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Cyn d I is presented as a dominant 32 kd band and a minor 29 kd band, both binding IgE. Both bands are identified by monospecific rabbit antibodies (Abs) raised against Cyn d I. These Abs only weakly precipitate allergens from other grass species, indicating that Cyn d I possesses some unique immunochemical properties. Two of four purified murine monoclonal Abs raised against Cyn d I also bind to both bands of Cyn d I, indicating that the bands represent isoallergens with slightly different immunochemical properties. All four monoclonal Abs cross-react with pollen components from other grass species, especially Poa pratensis and Dactylis glomerata. The NH₂-terminal sequence corresponding to approximately 10% of the complete sequence was determined, and it revealed high homology to the corresponding sequence of the major allergen of Lolium perenne, Lol p I. From the amino acid composition determination and immunoelectrophoretic comparison, the amount of Cyn d I in the source whole-pollen extract was estimated to be 15% wt/wt. (J ALLERGY CLIN IMMUNOL 1991;88:763-74.)

Key words: Grass, pollen, allergen, characterization, purification, *Cynodon dactylon*, Cyn d I, monoclonal antibodies

The allergenic composition of *Cynodon dactylon* (Bermuda grass) pollen extracts has been investigated in several studies.¹⁻⁵ In different populations of grass-allergic individuals, the importance of one allergenic component in the extract was noticed. In an early study with gel filtration and IEC, Orren and Dowdle¹ isolated an allergenic fraction that contained a 30 kd protein (BGP-1) and elicited a skin reaction in all studied South African subjects sensitive to the crude extract. With immunoblotting, Ford and Baldo² identified a 34 kd component (component 9) that bound IgE in all 44 investigated Australian sera, and in a

similar study, Shen et al.³ identified a 32 kd component that bound IgE in 16 of 21 Chinese sera. In an immunoelectrophoretic study, we identified a 32 kd allergen (Ag 34/35) that bound IgE in 31 of 32 sera from *Cynodon dactylon*-sensitized American individuals,⁴ and in another similar study, this allergen was demonstrated to bind IgE in all sera from 20 Danish grass-allergic subjects.⁵

We present, in this study, a procedure to isolate this major allergen of Bermuda-grass pollen, Cyn d I, and a study of physicochemical and immunochemical characterization. Short accounts of this work have been published elsewhere.^{6, 7}

MATERIAL AND METHODS

Pollen extracts

Pollens from *Cynodon dactylon*, 10 other grasses, and birch were obtained from Allergon AB (Uppsala, Sweden) as dried defatted material. According to the manufacturer's certificates, the *Cynodon dactylon* pollen samples contained approximately 99% pure pollen grains and approximately

From *ALK Research, Hørsholm, Denmark, and **Department of Pediatrics, University of Arizona, Tucson, Ariz.

Received for publication March 6, 1991.

Revised June 13, 1991.

Accepted for publication June 17, 1991.

Reprint requests: Finn Matthiesen, ALK Research, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark.

1/1/31931

Abbreviations used

<i>Cynd</i> :	<i>Cynodon dactylon</i> (Bermuda grass) pollen extract
<i>Alop</i> :	<i>Alopecurus pratensis</i> (foxtail) pollen extract
<i>Dacg</i> :	<i>Dactylis glomerata</i> (orchard grass) pollen extract
<i>Holl</i> :	<i>Holcus lanatus</i> (velvet grass) pollen extract
<i>Lolp</i> :	<i>Lolium perenne</i> (ryegrass) pollen extract
<i>Pasn</i> :	<i>Paspalum notatum</i> (Bahia grass) pollen extract
<i>Phlp</i> :	<i>Phleum pratense</i> (timothy) pollen extract
<i>Phrc</i> :	<i>Phragmites communis</i> (reed) pollen extract
<i>Poap</i> :	<i>Poa pratensis</i> (June grass) pollen extract
<i>Secc</i> :	<i>Secale cereale</i> (cultivated rye) pollen extract
<i>Sorh</i> :	<i>Sorghum halepense</i> (Johnson grass) pollen extract
<i>Betv</i> :	<i>Betula verrucosa</i> (birch) pollen extract
<i>Cyn d I</i> :	Group I allergen of <i>Cynd</i> pollen
<i>Lol p I</i> :	Group I allergen of <i>Lolp</i> pollen
anti- <i>Cynd</i> :	Polyspecific rabbit antibodies against <i>Cynd</i>
anti- <i>Cyn d I</i> :	Monospecific rabbit antibodies against <i>Cyn d I</i>
anti- <i>Lol p I</i> :	Monospecific rabbit antibodies against <i>Lol p I</i>
Ab:	Antibody
MAB:	Monoclonal antibody
<i>CdI1</i> , <i>CdI2</i> ,	Monoclonal antibodies against
<i>CdI3</i> , <i>CdI4</i> :	<i>Cyn d I</i>
Con A:	Concanavalin A
pI:	Isoelectric point
MW:	Molecular weight
RIE:	Rocket immunoelectrophoresis
RRIE:	Rocket radioimmunoelectrophoresis
IE:	Immunoelectrophoresis
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
FRIE:	Fused rocket immunoelectrophoresis
LIA:	Luminescence immunoassay
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IEC:	Ion exchange chromatography
CM:	Carboxymethyl
PMP:	Paramagnetic particles

1% other plant parts from the flowers of the same grass. Detectable foreign pollens constituted <0.5%. The pollens were stored dry at -20°C until use and extracted as described earlier.^{4,5}

The pollen extracts from plant species other than *Cynodon dactylon* were prepared in an identical way for use in cross-reactivity studies. The studied plant species and the corresponding extract names are presented in the abbreviation list.

Polyclonal rabbit Abs

Polyspecific rabbit Abs raised against *Cynd*, anti-*Cynd*, were the same as in an earlier study.⁴ Monospecific Abs against *Cyn d I* were raised by immunizing two rabbits, each receiving a monthly injection of 1 μg of purified allergen in Freund's incomplete adjuvant; anti-*Cyn d I* Abs were purified from serum after three bleedings.^{5,8} Monospecific Abs against *Lol p I*, anti-*Lol p I*, were prepared in a similar way, as reported earlier.⁵

Murine MAbs

Four MAbs, *CdI1*, *CdI3*, and *CdI4*, were obtained after immunization of one BALB/c mouse according to the following immunization protocol: intraperitoneal immunization with 50 μg of *Cyn d I* adsorbed to 100 μg of Al(OH)₃, was performed three times. One month later, the mouse was boosted intraperitoneally, and 3 days later, the spleen was removed. Spleen cells were fused with X-63 Ag 8.6.5.3 myeloma cells essentially as described by Köhler and Milstein.⁹

The cultures were grown in RPMI 1640 with 10% fetal calf serum. Supernatants were tested by ELISA for reactivity toward *Cyn d I* with anti-*Cyn d I*-coupled *Cyn d I* as catching layer and alkaline phosphatase-labeled antimouse immunoglobulin (D314, Dakopatts A/S, Glostrup, Denmark) as detection layer. Hybrids reacting positively were cloned and recloned by limiting dilution until all wells derived from single cells were positive.

MAbs were affinity purified from ascites on protein G columns (Pharmacia Diagnostics, Uppsala, Sweden) by elution with 0.1 mol/L of glycine buffer (pH 11), as described by the manufacturer. The purity of the Abs were tested in the PhastSystem technique (Pharmacia Diagnostics) by isoelectric focusing in polyacrylamide gels according to the manufacturer. Subclasses were determined by the Ouchterlony technique¹⁰ with rabbit antimurine Abs against IgM Fc fraction, IgA Fc fraction, IgG1, IgG2_{ab}, and IgG3 (Nordic Immunology, Tilburg, The Netherlands). The four MAbs were characterized as follows: *CdI1* (IgG1), pI $\approx$ 7.2; *CdI2* (IgG1), pI $\approx$ 8.5; *CdI3* (IgG1), pI $\approx$ 6.6; and *CdI4* (IgG1), pI $\approx$ 7.4.

The concentration of MAbs in the protein G affinity-purified fraction was estimated by ultraviolet absorption at 280 nm and interpolation on a standard curve prepared from similar measurements of bovine IgG (Sigma Chemical Co., St. Louis, Mo.) in seven concentrations from 10 $\mu\text{g}/\text{ml}$ to 10 mg/ml.

Serum pools and skin testing

A serum pool consisting of sera from 32 American grass pollen-sensitized individuals⁴ was used for allergen detection after IE and immunoblotting and used in an IgE-inhibition experiment.

Skin prick tests were performed with seven grass-pollen extracts: *Cynd*, *Cyn d* I, and pollen extracts of *Bromus*, *Bouteloua*, *Lolium*, *Phleum*, and *Sorghum* spp (1:20 wt/vol in 50% glycerol, Hollister-Stier Laboratories, Spokane, Wash.). The amount of *Cyn d* I in *Cynd* and the *Cyn d* I preparation were equilibrated in RIE with anti-*Cyn d* I. Skin tests were performed on 49 patients resident in Arizona, who were probably allergic to grass pollens on the basis of clinical criteria. All patients had a wheal response of at least 5 mm to histamine phosphate (0.55 mg/ml in test solution).

IE and SDS-PAGE

Analyses with CIE, CRIE, RIE, RRIE, and FRIE were performed at pH 8.6, with 1% agarose gels (type HLA, Lítex, Copenhagen, Denmark) as described earlier.^{4,11} Electrophoresis in SDS-PAGE, electroblotting into nitrocellulose paper, and IgE immunoblotting were performed exactly as described earlier.^{5,8}

Detection of specificity of rabbit anti-*Cyn d* I on SDS-PAGE electroblots (IgG immunoblotting) was performed as follows: electroblots were incubated overnight with 0.5 μ l of rabbit antiserum diluted 1:100,000 vol/vol in incubation buffer (0.05% Tween 20 in 0.05 mol/L of Tris and 0.15 mol/L of NaCl, adjusted to pH 10.3), followed by three washings in incubation buffer, followed by a 3-hour incubation in alkaline phosphatase-conjugated swine antirabbit IgG Abs (D306, Dakopatts A/S) diluted 1:1000 vol/vol in incubation buffer. The blots were developed by use of freshly prepared alkaline-phosphatase substrate (0.11 mmol/L of nitroblue tetrazolium, 0.14 mmol/L of 5-bromo-4-chloro-3-indolylphosphate, and 3.6 mmol/L of MgCl₂ in 0.1 mol/L of ethanolamine buffer, adjusted to pH 9.6).

Detection of specificity of murine MABs on SDS-PAGE electroblots was performed as for rabbit antisera, however, with the following changes: the amount of Abs were adjusted to 20 μ g per sample, and detection of binding was done with alkaline phosphatase-conjugated rabbit antimurine immunoglobulin (D314, Dakopatts A/S), diluted 1:1000 vol/vol in incubation buffer.

MW markers were composed of some of the following proteins: *Escherichia coli* β -galactosidase subunit, 116 kD; rabbit muscle phosphorylase b subunit, 97 kD; bovine serum albumin, 66 kD; ovalbumin, 43 kD; rabbit glyceraldehyde-3-phosphate-dehydrogenase, 36 kD; bovine erythrocyte carbonic anhydrase, 29 kD; soybean trypsin inhibitor, 20 kD; chicken-egg lysozyme, 14.4 kD; α -lactoglobulin, 14.2 kD (all from Sigma Chemical Co.).

Purification of *Cyn d* I

Nine batches of *Cyn d* I were purified from *Cynd* by a combination of Con A-Sepharose chromatography and IEC.

Pollen extract (between 30 and 300 mg of *Cynd*), dissolved in acetate buffer (pH 5.0; I = 0.05), was applied on a column with Con A-Sepharose (Pharmacia Diagnostics, 8 mg of Con A per milliliter of gel; gel volume, 0.1 ml/mg of extract). The extract was eluted with methyl- α -D-glucopyranoside (Sigma Chemical Co.) in two or three steps of succeeding concentrations (0.001, 0.005, and 0.100 mol/L wt/vol in acetate buffer, pH 5.0, with 0.001 mol/L MnCl₂, MgCl₂, and CaCl₂); first step might be omitted (Fig. 1). In each step, approximately four column volumes were applied, and fractions of 1 to 5 ml were collected and investigated in FRIE. The 0.005 mol/L fraction containing *Cyn d* I was pooled, dialyzed, and lyophilized.

This *Cyn d* I-enriched fraction, corresponding to 10% to 15% wt/wt of the applied pollen extract, was dissolved in acetate buffer (pH 5.0; I = 0.05) and applied on a CM-Sepharose column (Pharmacia Diagnostics; gel volume, 0.3 ml/mg of enriched extract). Elution of extract was performed by increasing the ionic strength with acetate from 0.05 to 1.00 in variable steps (Fig. 1). Fractions of 1 ml were collected and tested in FRIE. Fractions eluted with ionic strength 0.010 to 0.015 contained *Cyn d* I and were pooled, dialyzed, and lyophilized. In this fashion, an amount of 1.0% to 1.5% wt/wt of the applied pollen extract was isolated as *Cyn d* I and used for Ab manufacturing, inhibition experiments, and physicochemical analysis.

LIA-inhibition experiment

Test of allergenicity of *Cyn d* I was done with an LIA for specific IgE (Ciba Corning Diagnostics, Boston, Mass.), earlier described in principle.⁸

Briefly, *Cynd* extract was covalently coupled to microscopic PMPs and used as solid phase in an inhibition experiment. Serum pool (25 μ l, 1:4 dilution in water) was inhibited with *Cynd* (25 μ l, concentrations ranging from 1000 to 0.001 μ g/ml) or with equal amounts of purified *Cyn d* I (as measured in RIE with anti-*Cyn d* I) and incubated with *Cynd*-coated PMP. Free and bound IgE were magnetically separated, and noninhibited IgE was detected in a luminometer (Magic Lite analyzer, Ciba Corning Diagnostics) with a monoclonal anti-IgE (ALK Laboratories, Hørsholm, Denmark) coupled to the chemiluminescence reagent, acridinium ester.¹²

NH₂-terminal sequence

The NH₂-terminal amino acid sequence of the affinity-purified and lyophilized *Cyn d* I was determined on a 477 pulsed liquid-phase protein/peptide sequencer in combination with a 120A high-performance liquid chromatography on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, Calif.) according to the method of Hunkapillar et al.¹³

The amino acid composition was determined after derivatization with o-phthalaldehyde, as described by Barkholt and Jensen¹⁴; prevention of reduction of cysteine during hydrolysis was done by derivatization with 3,3'-dithiopropionic acid. The content of tryptophane was not determined.

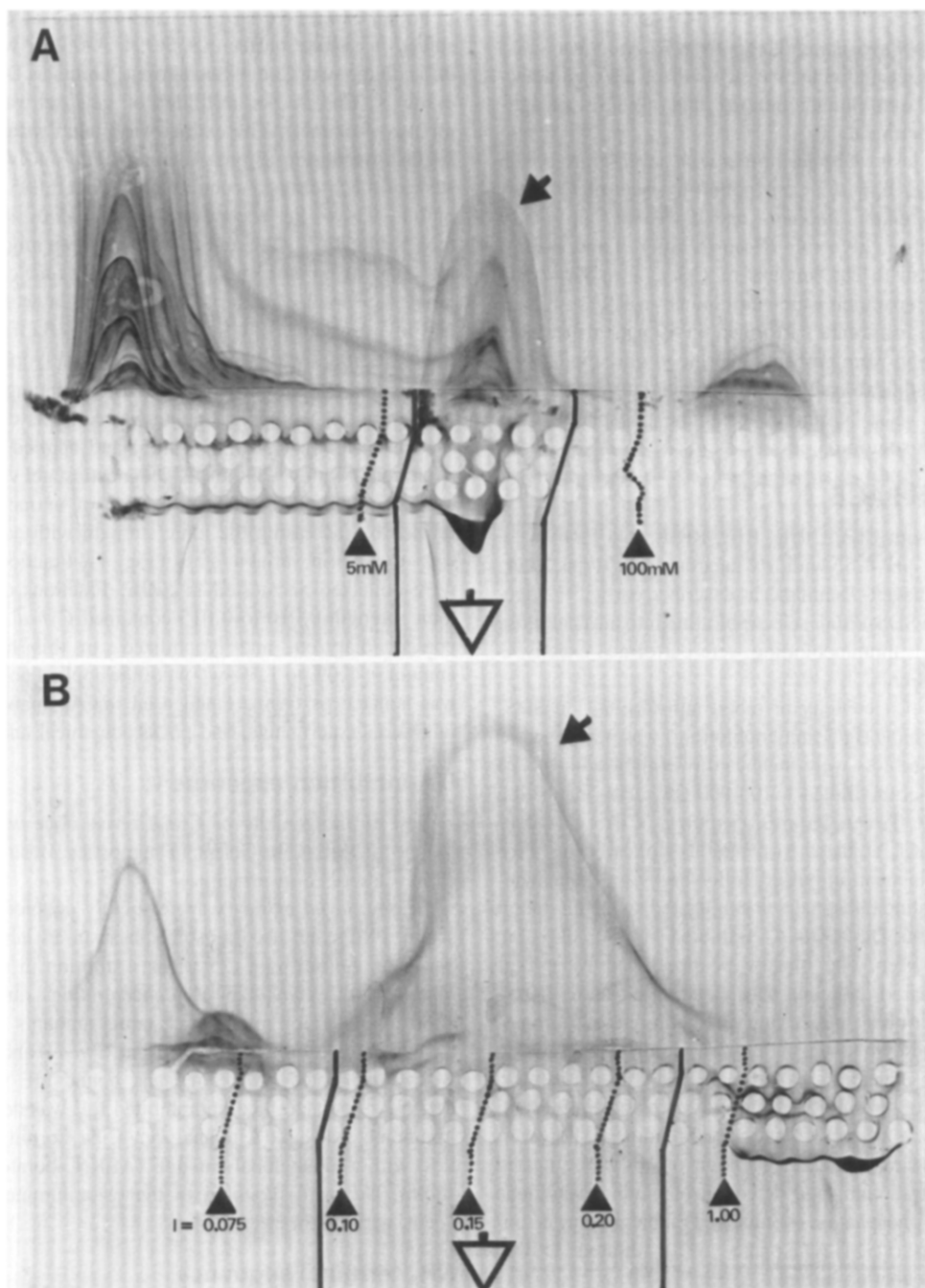


FIG. 1. Two-step purification of *Cyn d I*. **A**, FRIE analysis of 84 fractions from first-step column (Con A-Sepharose). **B**, FRIE analysis of 84 fractions from second-step column (CM-Sepharose); changes of elution buffers ($\blacktriangle$); concentrations of methyl- α -D-glucopyranoside and ionic strength, respectively, as indicated. Fractions between *black solid lines* were pooled; *black arrows* indicate the *Cyn d I* precipitates. In both FRIE, 14 μ l of each fraction was applied in wells against 8.3 μ l/cm² of anti-*Cyn d*; electrophoresis for 16 hours at 2 V/cm after fractions were allowed to fuse for 1 hour.

RESULTS

An allergen, *Cyn d I*, has been isolated from *Cyn d* pollen by a combination of Con A affinity chromatography and IEC (Fig. 1). The purity was

examined in CIE/CRIE and in SDS-PAGE electrophoresis.

With Abs raised against whole pollen extract (anti-*Cyn d*), the purified *Cyn d I* is revealed as a well-

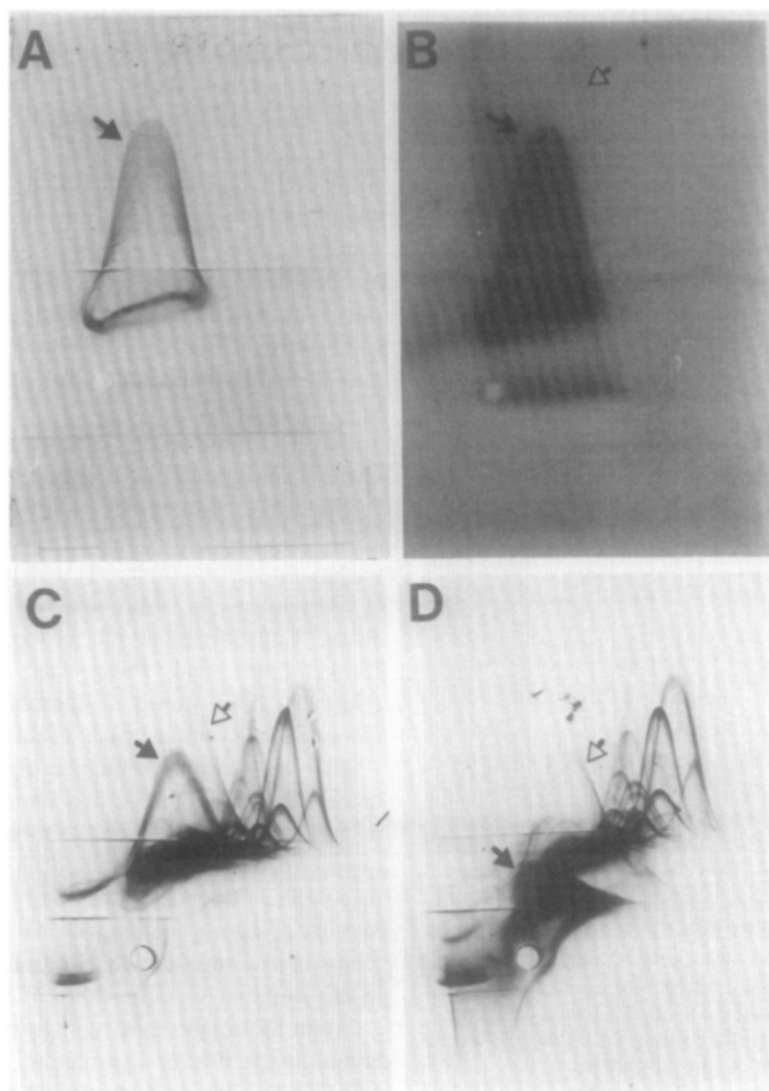


FIG. 2. Examination of purity and IgE-binding capacity of *Cyn d* I in CIE/CRIE. **A**, CIE with 15 μ g of *Cyn d* I against 12.8 μ l/cm² of anti-*Cynd*. **B**, CRIE, autoradiography of **A**. **C**, CIE with 75 μ g of *Cynd* against 12.8 μ l/cm² of anti-*Cynd*. **D**, CIE as in **C**, but with 20 μ l/cm² of anti-*Cyn d* I in the intermediate gel. *Black arrows* illustrate proposed 32 kd component of *Cyn d* I; *white arrows* illustrate proposed 29 kd component; first dimension electrophoresis for 30 minutes at 10 V/cm; second dimension electrophoresis overnight at 2 V/cm.

defined IgE-binding anodic precipitate in CIE/CRIE (Fig. 2). In CRIE, a weak IgE binding contaminant with the same migration is also observed; this precipitate is not blue stained in CIE. In SDS-PAGE gold-stained electroblots, the prepared *Cyn d* I is revealed as a major 32 kd band with a minor band at 29 kd (Fig. 3). Artifact bands from oxidized dithiotreitol in the buffer are also present.¹⁵ The 32 kd band corresponds to the dominant band in *Cynd*, and it binds IgE in a serum pool from grass pollen-allergic individuals.

The IgE-binding properties of *Cyn d* I was also examined in an IgE-inhibition experiment (Fig. 4).

In this modified LIA with *Cynd* as solid phase, IgE from a serum pool of *Cynd*-sensitized individuals was inhibited with *Cynd* and *Cyn d* I (Fig. 4). *Cyn d* I was examined in amounts corresponding to the amounts of *Cyn d* I in *Cynd*, as estimated in RIE. The inhibition capacity of the *Cyn d* I preparation was 80% of the inhibition capacity of *Cynd*, as judged after parameter estimation (SigmaPlot version 4.04, Jandel Scientific, Corte Madera, Calif.).

The same two preparations were also investigated by skin testing of proposed grass pollen-sensitized individuals from Arizona. Of 49 investigated patients, 30 responded to at least one grass-pollen extract, 27

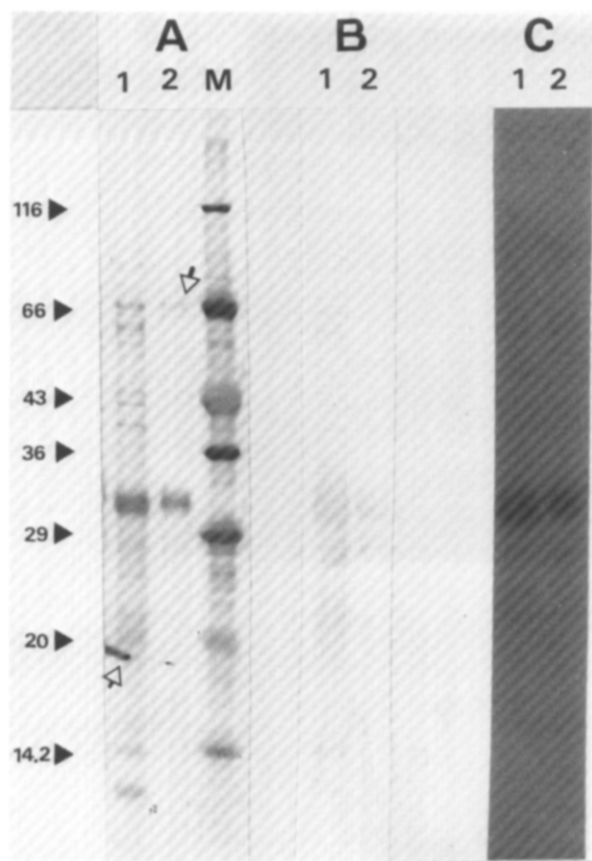


FIG. 3. Examination of purity and verification of IgE binding capacity of *Cyn d I* in SDS-PAGE. **A**, Gold-stained blot. **B**, IgG immunoblot with anti-*Cynd*. **C**, IgE immunoblot with pool of sera. Applied probes: 1, 3 μ g of *Cynd*; 2, 0.5 μ g of *Cyn d I*; M, Mixture of markers. White arrows identify a gold-blotting artifact and a band at 66 kd, which is due to the reduction reagent in sample buffer.

responded to *Cynd*, and 20 patients responded to the *Cyn d I* preparation (Table I). Wheal responses of at least 5 mm were regarded as positive responses. All patients that responded to *Cyn d I* also responded to *Cynd*. The responses to *Cyn d I* were always smaller than responses to whole-pollen extract, and there is a statistically significant relationship between responsiveness to *Cynd* and to *Cyn d I* ($p = 0.0003$).

Monospecific rabbit Abs against *Cyn d I*, anti-*Cyn d I*, identify the major 32 kd allergen from *Cynd* pollen from previous immunoelectrophoretic studies^{4, 5, 16} (Fig. 2). Cross-reactivity of anti-*Cyn d I* to components in pollen extracts from other grasses than *Cynd* was investigated in RIE (Fig. 5). Although *Cyn d I* is easily precipitated with anti-*Cyn d I*, similar allergens from the other grass pollens are only very weakly precipitated. The strongest cross-reactivity was detected to components in *Dacg*, *Lolp*, *Phlp*, and *Poap*. In comparison, similar monospecific rabbit Abs against *Lol p I*, anti-*Lol p I*,⁵ easily precipitates

allergenic components from other grasses. However, anti-*Lol p I* does not precipitate *Cyn d I*.

Four murine MAbs, *CdI1*, *CdI2*, *CdI3*, and *CdI4*, were also raised against *Cyn d I*. Together with rabbit anti-*Cyn d I*, these Abs were studied in SDS-PAGE IgG immunoblotting for examination of cross-reactivity to pollen proteins from other grass species (Table II). Anti-*Cyn d I* and the four MAbs all identify the major allergenic component of *Cynd* pollen from earlier immunoblot studies.^{2, 3, 5} Although *CdI1* and *CdI2* only identify this 32 kd component, anti-*Cyn d I*, *CdI3*, and *CdI4* also bind to the 29 kd component (Fig. 6).

In IgG immunoblots, all the Abs raised against *Cyn d I* bind group I components from other grasses. Rabbit anti-*Cyn d I* binds group I components (MW 28 to 35 kd) in all studied extracts with the possible exception of *Phlp* (Table II). *CdI1* binds to the group I components in *Alop*, *Dacg*, *Pasn*, *Phrc*, and *Poap*; *CdI2* binds to the group I components in *Dacg*, *Lolp*, and *Poap*; and *CdI3* and *CdI4* (Fig. 6) bind group I components from *Alop*, *Dacg*, *Phlp*, *Paop* and *Secc* (Table II).

The NH₂-terminal sequence of *Cyn d I* has been determined. The 27 identified amino acids correspond to approximately 10% of the molecule, and the sequence reveals a high degree of homology to the corresponding sequence of the group I allergen of *Lolium perenne* (ryegrass) pollen, *Lol p I*^{17, 18} (Table III). The amino acid composition also reveals close similarities to the amino acid composition of *Lol p I* (Table IV).

From the amino acid composition determination and a comparison of *Cynd* and *Cyn d I* in RIE with anti-*Cyn d I*, the amount of *Cyn d I* in whole-pollen extract was estimated to be 15% wt/wt.

DISCUSSION

The occurrence of sensitization to Bermuda-grass pollen in the subtropical regions of the world is well established.¹⁹ In populations of grass pollen-sensitized individuals from South Africa,¹ Australia,² Taiwan,³ and the United States,⁴ the allergenic activity of single Bermuda grass-pollen components has been documented. In these studies, the importance of a 30 to 34 kd allergen was established, and the purification of this allergen, *Cyn d I*, has been the aim of this study.

After a screening of different affinity chromatography techniques, Con A-Sepharose affinity chromatography in combination with CM-Sepharose chromatography was found to be useful in purification of *Cynodon I*. The affinity of *Cynodon*-pollen components to Con A has also been noticed elsewhere. Watson et al.²⁰ found that Con A might differentiate between *Cynodon* pollen and most other grass pollens

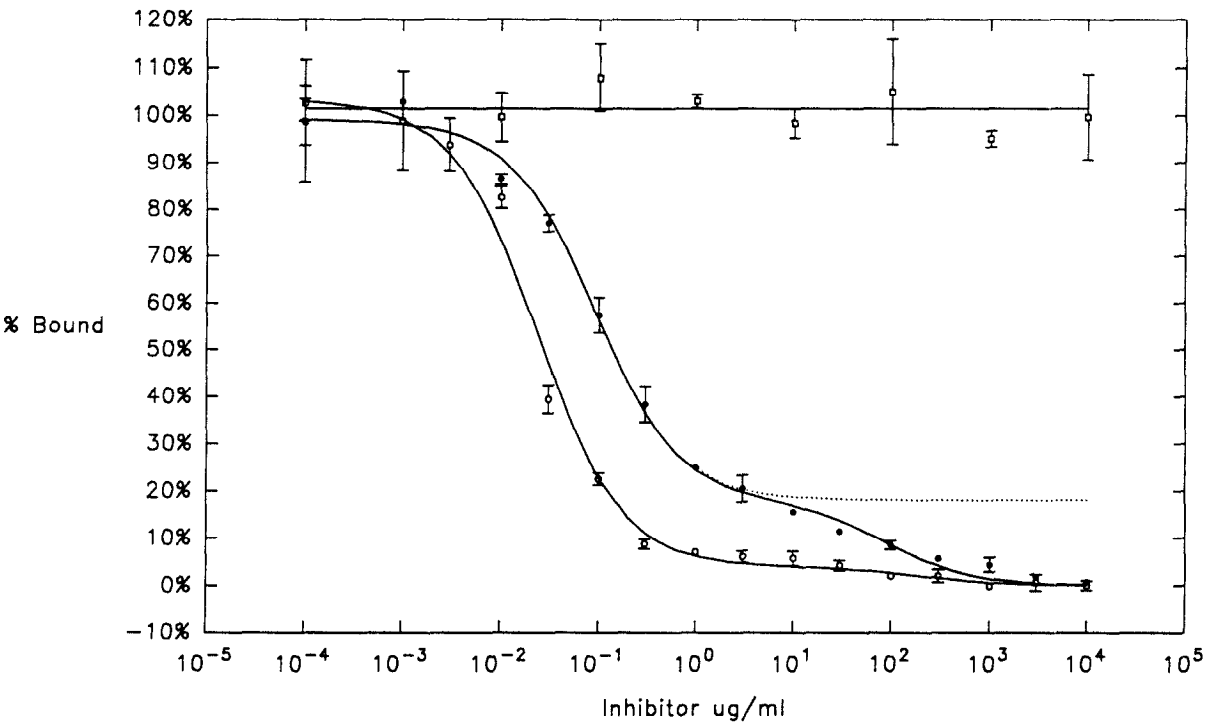


FIG. 4. Examination of allergenic potency of *Cyn d* I in LIA-inhibition experiment; *Cynd* as solid phase. Serum pool inhibited with whole-pollen extract *Cyn d* (○); purified *Cyn d* I (●); and *Dermatophagoides pteronyssinus* (□) (dust mite) extract as a negative control. Extract concentrations in micrograms per milliliter. *Cyn d* I concentration in purified fraction is approximated to *Cyn d* I concentration in pollen extract, as described in text (*Cyn d* I corresponds to approximately 15% of whole-pollen extract *Cynd*). Each point corresponds to the mean of a triple determination (standard deviations indicated by lines); dotted line is the estimated inhibition curve (SigmaPlot version, 4.04).

TABLE I. Skin test results of a group of 30 patients who responded to at least one of seven grass-pollen extracts, including *Cynd* and the *Cyn d* I preparation

	<i>Cynd</i> wheal size (mm)					Total No.
	<5	5-9	10-14	15-19	>20	
<i>Cyn d</i> I wheal size (mm)						
<5	3	5	0	2	0	10 (neg)
5-9	0	5	6	3	0	20 (pos)
10-14	0	1	1	0	4	
Total No.	3 (neg)	27 (pos)				30

Neg, Negative; pos, positive.
Only wheal responses of 5 mm or more were regarded as positive responses. The amount of *Cyn d* I in the *Cynodon dactylon* extracts were equilibrated in CIE before testing.

with importance in allergy caused by the specific binding of Con A to wall components in *Cynodon* pollen, and Karlstam and Nilsson²¹ found that in nine grass pollens with importance in allergy, only *Cynodon* and *Phragmites* pollens revealed allergens that bound to

Con A. For example, in both studies, the binding of *Lolium*- or *Phleum*-pollen components were not observed.

Obviously, *Cyn d* I binds the lectin, Con A, whereas other major grass-pollen allergens, like *Lol p* I and

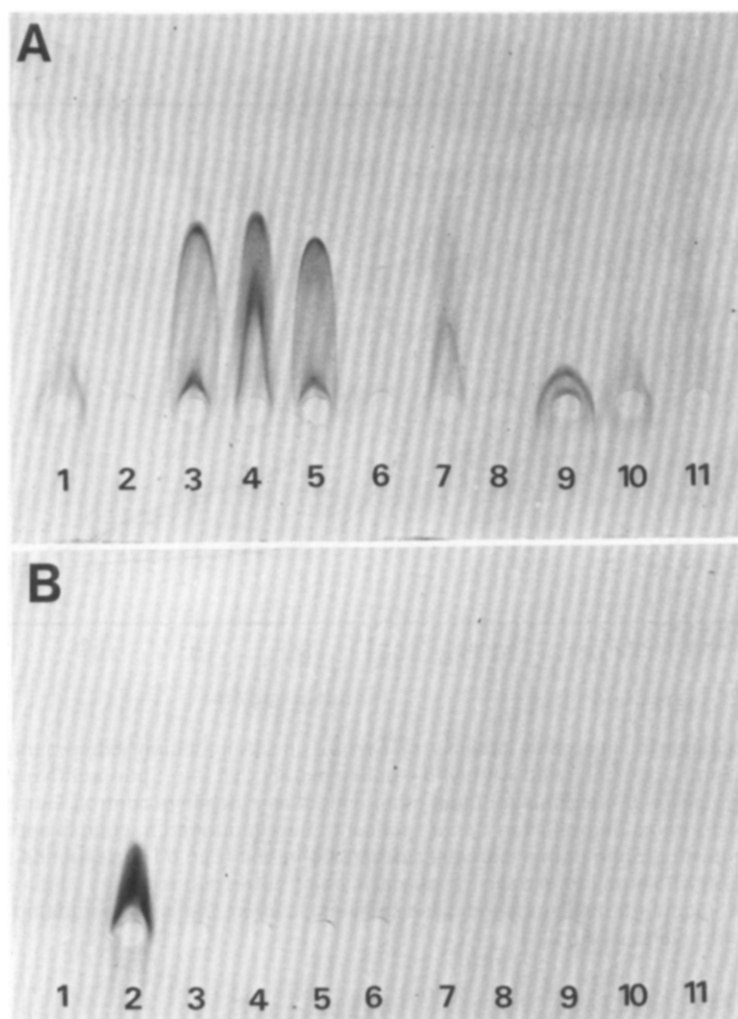


FIG. 5. Cross-reactivity of rabbit polyclonal Abs against *Lol p I* and *Cyn d I* in RIE. **A**, Anti-*Lol p I*. **B**, Anti-*Cyn d I*. Applied in the wells were 10 μ g of each of following grass-pollen extracts: 1, *Alop*; 2, *Cynd*; 3, *Dacg*; 4, *Holl*; 5, *Lolp*; 6, *Pasn*; 7, *Phlp*; 8, *Phrc*; 9, *Poap*; 10, *Secc*; and 11, *Sorh*; electrophoresis against 2.9 μ l/cm² of monospecific Abs.

Phl p V, do not. This finding indicates that *Cyn d I* contains carbohydrate with free terminal α -D-mannopyranoside or α -D-glucopyranoside residues, which is not present or accessible in other major allergens.

Both rabbit polyclonal monospecific Abs (anti-*Cyn d I*) and murine MAbs (*CdI1*, *CdI2*, *CdI3*, and *CdI4*) were raised against the purified allergenic fraction. They were demonstrated to react specifically against *Cyn d I* in CIE (Fig. 2) and SDS-PAGE IgG immunoblots (Table II; Fig. 6). These Abs were used in the additional characterization of the allergen.

The purity of the isolated *Cyn d I* was examined in CIE/CRIE and SDS-PAGE. By both techniques, more than one component is found in the isolated fraction. Two components of 32 kd and (much less dominant) 29 kd are present and reveal similar immunochemical

characteristics, as observed in SDS-PAGE immunoblots with anti-*Cyn d I* and the MAbs. Two MAbs (*CdI1* and *CdI2*) were able to distinguish between the 32 and 29 kd components. This finding indicates that they constitute isoallergenic forms of *Cyn d I* with slightly different immunochemical characteristics; the differences might be due to differences in amino acid chain lengths or to different degrees of glycosylation, which could affect the overall structure of the protein. Amino acid sequence determinations were not disrupted by impurities, indicating that they are not stoichiometric dominant.

The IgE-binding capacity of the purified *Cyn d I* was examined by four techniques with sera from grass pollen-sensitized individuals. In CRIE and in SDS-PAGE immunoblot, the IgE-binding capacity was

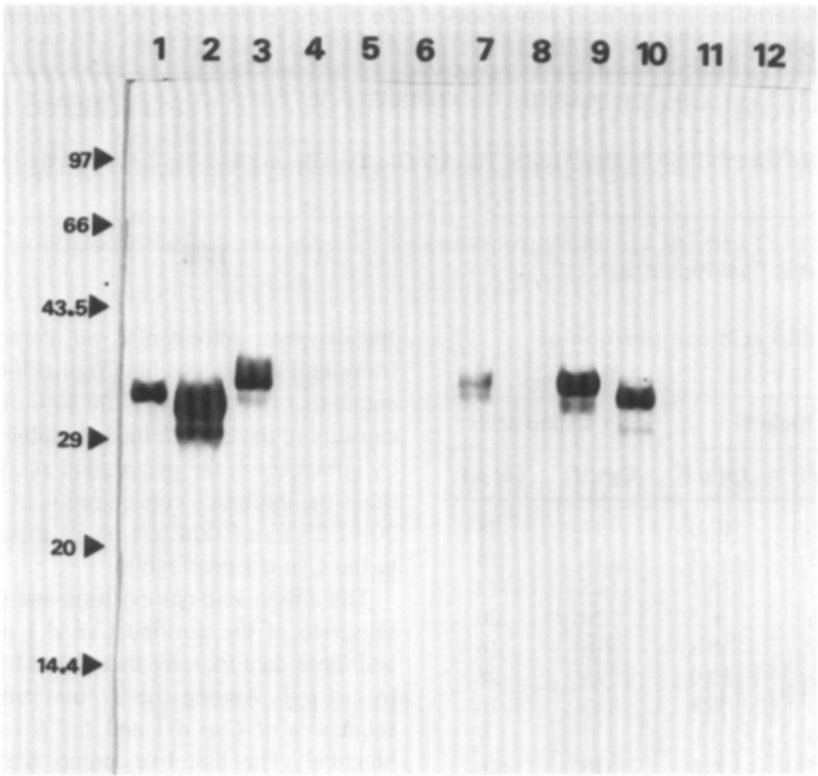


FIG. 6. Cross-reactivity of murine monoclonal anti-*Cyn d I* (*Cd14*) in SDS-PAGE IgG immunoblot; 5 μ g of each of the pollen extracts was applied in the slits. Sequence of grass-pollen extracts as in Fig. 5, however, with 12, *Betv*, added as negative control.

TABLE II. Cross-reactivity of anti-*Cyn d I* Abs to components in other grass pollens (and birch-pollen extract as reference), as observed in SDS-PAGE IgG immunoblots

Pollen extract	MW (kd) SDS-PAGE	Anti- <i>Cyn d I</i> (rabbit Ab)	<i>Cd11</i>	<i>Cd12</i>	<i>Cd13</i>	<i>Cd14</i>
			(MAbs)			
<i>Alop</i>	33	+++	+	—	+++	+++
<i>Cynd</i>	32	+++	+++	+++	+++	+++
	29	+	—	—	+++	+++
<i>Dacg</i>	35	+++	+	+++	+++	+++
	34	+++	+	+++	+++	+++
<i>Holl</i>	34	+++	—	—	—	—
<i>Lolp</i>	34	+++	—	+++	—	—
<i>Pasn</i>	32	+++	+++	—	—	—
	28	+	+++	—	—	—
<i>Phlp</i>	34	+	—	—	(+)	+
<i>Phrc</i>	32	+++	+	—	—	—
<i>Poap</i>	34	+++	+++	+++	+++	+++
	32	+	+++	—	—	+
<i>Secc</i>	33	+++	—	—	+	+++
	29	—	—	—	—	+
<i>Sorh</i>	32	+	—	—	—	—
<i>Betv</i>	—	—	—	—	—	—

+++ , Strong binding; + , weak binding; (+) , very weak binding; — , no binding detected.
The molecular weight of anti-*Cyn d I* recognized components are presented. See also Fig. 6.

TABLE III. The NH₂-terminal amino acid sequence of *Cyn d* I and corresponding NH₂-terminal sequences of *Lol p* I

<i>Cyn d</i> I: Ala-Met-Gly-Asp-Lys-Pro-Gly-Pro-X-Ile-Thr-Ala-Thr-Tyr-Gly-Asp-Lys-Trp-Leu-Asp-Ala-Lys-Ala-Thr-Phe-Tyr-Gly
<i>Lol p</i> I: Ile-Ala-Lys-Val-Pro-Pro-Gly-Pro-Asn-Ile-Thr-Ala-Glu-Tyr-Gly-Asp-Lys-Trp-Leu-Asp-Ala-Lys-Ser-Thr-Trp-Tyr-Gly

X, In the sequence of *Cyn d* I represents a proposed glycosylation site (presumably Asn). Sequence homologies are underlined.

*According to Cottam et al.¹⁷ and Perez et al.¹⁸.

TABLE IV. The amino acid composition of *Cyn d* I*

Amino acid	Mole %		No. amino acids	
	<i>Cyn d</i> I	<i>Lol p</i> I	<i>Cyn d</i> I	<i>Lol p</i> I
Asp/Asn	11.6	12.0	27	28
Thr	4.5	7.7	11	18
Ser	6.3	5.1	15	12
Glu/Gln	8.6	8.5	20	20
Pro	6.9	6.8	16	16
Gly	11.2	12.0	26	28
Ala	8.0	6.8	19	16
Cys	3.0	3.0	7	7
Val	4.4	6.8	10	16
Met	2.2	0.9	5	2
Ile	4.8	4.7	11	11
Leu	5.8	3.4	14	8
Tyr	4.0	3.8	9	9
Phe	3.8	3.4	9	8
His	2.1	1.3	5	3
Lys	10.3	11.1	24	26
Arg	2.4	2.6	6	6
Trp	(ND)	(+ 2.6)	(ND)	(6)
Total†	100.0	100.0	234	234

Asp/Asn, aspartic acid/asparagine; Thr, threonine; Ser, serine; Glu/Gln, glutamic acid/glutamine; Pro, proline; Gly, glycine; Ala, alanine; Cys, cysteine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; His, histidine; Lys, lysine; Arg, arginine; Trp, tryptophane; ND, not done.

Tryptophane was not determined. The numbers of specific amino acid residues in *Cyn d* I were estimated assuming that the total number of residues in *Cyn d* I and *Lol p* I are the same (N = 240).

*The corresponding amino acid composition of recombinant *Lol p* I clone 1A according to the method of Perez et al.¹⁸ is presented for comparison.

†Excluding tryptophane.

qualitatively verified, and in an IgE-inhibition assay, the inhibition capacity of the purified *Cyn d* I relatively to whole-pollen extract, *Cynd*, was quantitatively examined. The in vivo activity of the purified *Cyn d* I was examined by skin testing.

Both the IgE-inhibition assay and the skin testing indicate that *Cyn d* I possesses an important part of

the allergenic activity of *Cynd*. Parameter estimations from inhibition curve fits (SigmaPlot version 4.0) indicate that *Cyn d* I constitute 80% of the IgE-binding capacity of the extract; the remaining 20% is inhibited by impurities in the preparation at concentrations 200 times the inhibition concentration of *Cyn d* I. In vivo, 20 of 27 *Cynd* skin test positive patients responded to the *Cyn d* I preparation.

The amino acid composition and the NH₂-terminal sequence of the purified *Cyn d* I were determined, and these data indicate that *Cyn d* I is closely related to *Lol p* I. Among the 27 first amino acids in the sequences of *Cyn d* I and *Lol p* I, 12 residues are identical. The first five amino acids, however, are different, possibly caused by a higher genetic variance in the outermost NH₂-terminal sequence that might not be important for the overall structure of the allergen. In residue No. 9 of *Cyn d* I, a potential glycosylation site is present, as indicated in the sequencer chromatograms, and this residue corresponds to an asparagine residue in *Lol p* I; thus, a potential N-glycosylation site is present here.

As indicated by the NH₂-terminal sequence, the MW of 32 to 34 kD¹⁻⁵ and the pI of 6.0 to 6.8,⁴ physicochemical similarities of *Cyn d* I and other well-characterized group I allergens are evident. However, the presence of immunochemical similarities is more puzzling.

In earlier studies of the allergenic activity of whole-pollen extracts, extracts of *Cynodon* pollen were demonstrated to be poor inhibitors of IgE against other grasses,²²⁻²⁵ with the possible exceptions of *Bouteloua* and *Distichlis* spp.²⁴ In immunoprecipitation studies, rabbit antisera raised against whole-pollen extracts of temperate grasses did not (or only very weakly) precipitate components of *Cynodon* pollen,^{26, 27} and studies with rabbit antisera raised against *Cynodon*-pollen extracts^{27, 28} demonstrated that these only very weakly (if not at all) did bind to pollen components of most other investigated grasses, with the interesting exceptions of *Sorghum* spp.²⁷ and *Elymus* and *Koeleria* spp.²⁸ In other earlier studies, MAbs raised against *Lol p* whole-pollen extract were not able to bind to components in *Cynodon* pollen.^{29, 30}

However, Standring et al.³⁰ did report binding of their rabbit anti-*Lol p* I (anti-R7) Abs to a 29 to 31 kd components in a *Cynodon*-pollen extract, and Kahn and Marsh³¹ have characterized anti-*Lol p* I MABs (Mab 3.2) that, although they bind very weakly, bind to a *Cynd*-pollen component in a radioimmunoassay.

The study presented here has demonstrated that cross-reactivity of anti-*Cyn d* I Abs (rabbit and murine) to group I allergens of other species is definitely present. The rabbit anti-*Cyn d* I Abs specifically bind all other investigated group I allergens (Table II), but the binding might be rather weak, as indicated by the precipitates in RIE compared to the corresponding *Cyn d* I precipitate (Fig. 5). Murine MABs also cross-react with other group I allergens, especially the group I allergens *Dac g* I and *Poa p* I, which were recognized by all four MABs by immunoblotting.

As recently reported,⁵ Danish grass-sensitized individuals reveal IgE that binds to *Cynodon* pollen extract components, even though *Cynodon dactylon* does not naturally occur in Denmark. Also, these observations strongly suggest that *Cyn d* I and group I allergens from temperate grasses possess common IgG and IgE epitopes. However, when these studies are compared to cross-reaction studies with temperate grasses, the cross-reactions between anti-*Cyn d* I and other grasses are comparatively weak (Fig. 5). Moreover, it is easy to prepare reagents against other group I allergens that do not, or only very weakly, recognize *Cyn d* I.^{5, 31} In this study, anti-*Lol p* I Abs do not recognize *Cyn d* I in RIE (Fig. 5). This finding demonstrates that dominant rabbit IgG epitopes on *Lol p* I is not present on *Cyn d* I.

The observations might be explained as follows: *Cyn d* I and *Lol p* I possess some common epitopes, but *Lol p* I possesses additional epitopes not present on *Cyn d* I. When *Cyn d* I is used as an immunogen for preparation of both rabbit and murine Abs, non-dominant epitopes also present on *Lol p* I will be in effect; for example, in screenings for *Lol p* I-binding MABs, MABs identifying the dominant IgG epitopes of *Lol p* I will be more common, whereas in screenings for *Cyn d* I-binding MABs, MABs identifying the non-dominant epitopes of *Lol p* I (and *Cyn d* I) will be common.

However, specific epitopes only present on *Cyn d* I is not excluded. The *Cyn d* I precipitate after CIE is very dense (Fig. 5), and this finding is not the case for other group I allergens, as earlier reported.⁵ Thus, in general, rabbits respond more strongly against *Cyn d* I than *Lol p* I; it is assumed that this is due to the fact that (Danish) rabbits are not sensitized to the

subtropical grass through forage and therefore recognize *Cyn d* I-specific epitopes very effectively.

Because of the physicochemical and partly immunochemical similarities to *Lol p* I and other group I allergens, the purified allergen has been referred to throughout this article as *Cyn d* I. This should be in agreement with the nomenclature specified by the Allergen Standardization Subcommittee of the International Union of Immunochemical Societies.³²

We thank Ms. Lotte Friberg, Ms. Birgit Jacobsen, and Ms. Helle Friese Jensen for their skilful technical assistance through this study, Dr. Karen G. Welinder and Dr. Arne Jensen, Institute of Biochemical Genetics, the University of Copenhagen, for the sequence analyses and the amino acid composition determination and James Dover, MD, George Makol, MD, and Uwe Manthei, MD, for skin testing of their patients.

REFERENCES

1. Orren A, Dowdle EB. Studies on Bermuda-grass pollen allergens. *S Afr Med J* 1977;51:586-91.
2. Ford SA, Baldo BA. Identification of Bermuda grass (*Cynodon dactylon*)-pollen allergens by electroblotting. *J ALLERGY CLIN IMMUNOL* 1987;79:711-20.
3. Shen HD, Wang SR, Tang RB, Chang ZN, Han SH. Identification of allergens and antigens of Bermuda grass (*Cynodon dactylon*) pollen by immunoblot analysis. *Clin Allergy* 1988;18:401-9.
4. Matthiesen F, Schumacher MJ, Løwenstein H. An immunoelectrophoretic analysis of the allergens of *Cynodon dactylon* (Bermuda Grass) pollen. *J ALLERGY CLIN IMMUNOL* 1989;83:1124-34.
5. Matthiesen F, Løwenstein H. Group V allergens in grass pollens. II. An investigation of group V allergens in pollens from ten grasses. *Clin Exp Allergy* 1991;21:309-20.
6. Matthiesen F, Schumacher M, Løwenstein H. Characterization of the major allergen of *Cynodon dactylon* (Bermuda grass) pollen [Abstract]. *J ALLERGY CLIN IMMUNOL* 1988;81:266.
7. Matthiesen F, Klysner S, Løwenstein H. Monoclonal antibodies against group I and group V allergens of grass pollens [Abstract]. *Clin Exp Allergy* 1990;20(suppl 1):47.
8. Matthiesen F, Løwenstein H. Group V allergens in grass pollens. I. Purification and characterization of the group V allergen from *Phleum pratense* pollen, *Phl p* V. *Clin Exp Allergy* 1991;21:297-307.
9. Köhler G, Milstein C. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* 1975;256:495-7.
10. Outhrelony Ö, Nilsson LÅ. Immunodiffusion and immunoelectrophoresis. In: Weir DM, ed. *Handbook of experimental immunology*. Oxford: Blackwell Scientific, 1978:19.1-39.
11. Løwenstein H. Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Prog Allergy* 1978;25:1-62.
12. Weeks I, Beheshti I, McCapra F, Campbell AK, Woodhead JS. Acridinium esters as high specific-activity labels in immunoassay. *Clin Chem* 1983;29:1474-9.
13. Hunkapilliar MW, Hewick RM, Dreyer WJ, Hood LE. High-sensitivity sequencing with a gas-phase sequenator. *Methods Enzymol* 1983;91:399-413.
14. Barkolt V, Jensen AL. Amino acid analysis: determination of

- cysteine plus half cystine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive. *Anal Biochem* 1989;177:318-22.
15. Park KB, Labbé RG. Artifacts following a gold staining of Western-blotted membranes. *Anal Biochem* 1988;180:55-8.
 16. Baer H, Anderson MC, Helm RM, et al. The preparation and testing of the proposed international reference (IRP) Bermuda grass (*Cynodon dactylon*)-pollen extract. *J ALLERGY CLIN IMMUNOL* 1986;78:624-31.
 17. Cottam GP, Moran DM, Standring R. Physicochemical and immunochemical characterization of allergenic proteins from ryegrass (*Lolium perenne*) pollen prepared by a rapid and efficient purification. *Method Biochem J* 1986;234:305-10.
 18. Perez M, Ishioka GY, Walker LE, Chesnut RW. cDNA cloning and immunological characterization of the ryegrass-allergen *Lol p I*. *J Biol Chem* 1990;265:16210-5.
 19. Lewis WH, Viany P, Zenger VE. Airborne and allergenic pollen of North America. Boston: The Johns Hopkins University, 1983:105-27.
 20. Watson L, Knox RB, Creaser EH. Con A differentiates among grass pollens by binding specifically to wall glycoproteins and carbohydrates. *Nature* 1974;249:574-6.
 21. Karlstam B, Nilsson B. Characterization of grass pollen allergens by affinity chromatography on Concanavalin A-Sepharose. *J Immunol Methods* 1982;54:119-28.
 22. Bernstein IL, Perera M, Gallagher J, Michael JG, Johansson SGO. In vitro cross-allergenicity of major aeroallergenic pollens by the radioallergosorbent technique. *J ALLERGY CLIN IMMUNOL* 1976;57:141-52.
 23. Leiferman KM, Gleich GJ. The cross-reactivity of IgE antibodies with pollen allergens. I. Analysis of various species of grass pollens. *J ALLERGY CLIN IMMUNOL* 1976;58:129-39.
 24. Martin BG, Mansfield LE, Nelson HS. Cross-allergenicity among the grasses. *Ann Allergy* 1985;54:99-104.
 25. Schumacher MJ, Grabowski J, Wagner CM. Anti-Bermuda grass RAST binding minimally inhibited by pollen extracts from ten other grasses. *Ann Allergy* 1985;55:584-7.
 26. Marsh DG, Haddad ZH, Campbell DH. A new method for determining the distribution of allergenic fractions in biological materials: its application to grass-pollen extracts. *J ALLERGY* 1970;46:107-21.
 27. Watson L. Taxonomic patterns in grass pollen antigens and allergens, and their potential significance. *Proc Sydney Allergy Group* 1983;3:37-47.
 28. Schumacher MJ, Griffith RD, O'Rourke MK. Recognition of pollen and other particulate aeroantigens by immunoblot microscopy. *J ALLERGY CLIN IMMUNOL* 1988;82:608-16.
 29. Singh MB, Knox RB. Grass pollen allergens: antigenic relationships detected using monoclonal antibodies and dot blotting immunoassay. *Int Arch Allergy Appl Immunol* 1985;78:300-4.
 30. Standring R, Spackman V, Porter SJ. Distribution of a major allergen of ryegrass (*Lolium perenne*) pollen between other grass species. *Int Arch Allergy Appl Immunol* 1987;83:96-103.
 31. Kahn CR, Marsh DG. Monoclonal antibodies to the major *Lolium perenne* (ryegrass) pollen allergen, *Lol p I* (Rye I). *Mol Immunol* 1986;23:1281-8.
 32. Marsh DG, Goodfriend L, King TP, Løwenstein H, Platts-Mills TAE. Allergen nomenclature. *J ALLERGY CLIN IMMUNOL* 1987;80:639-45.

Bound volumes available to subscribers

Bound volumes of THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY are available to subscribers (only) for the 1991 issues from the Publisher, at a cost of \$49.00 for domestic, \$70.43 for Canadian, and \$67.00 for international subscribers for Vol. 87 (January-June) and Vol. 88 (July-December). Shipping charges are included. Each bound volume contains a subject and author index, and all advertising is removed. Copies are shipped within 30 days after publication of the last issue in the volume. The binding is durable buckram with the journal name, volume number, and year stamped in gold on the spine. *Payment must accompany all orders.* Contact Mosby-Year Book, Inc., Subscription Services, 11830 Westline Industrial Dr., St. Louis, MO 63146-3318; phone (800) 325-4177, ext. 4351, or (314) 453-4351.

Subscriptions must be in force to qualify. Bound volumes are not available in place of a regular journal subscription.