

Identification and characterization of epitopes on *Cyn d* I, the major allergen of Bermuda grass pollen

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Background: We identified three epitopes on *Cyn d* I by using four anti-*Cyn d* I monoclonal antibodies (MoAbs).

Methods: In a cross-inhibition binding assay, the binding of MoAbs 1-61 and 10-7 to *Cyn d* I was completely blocked by each other but not by MoAbs 4-37 and 11-7; the binding of MoAb 4-37 and MoAb 11-7 to *Cyn d* I was inhibited by themselves but not by other MoAbs. The epitope recognized by MoAbs 1-61 and 10-7 is designated as E1, and those recognized by MoAbs 4-37 and 11-7 are designated as E2 and E3, respectively.

Results: In a radioallergosorbent inhibition assay, we found that MoAbs 1-61 and 4-37 (1:50 diluted) can inhibit the binding of human Immunoglobulin Es to *Cyn d* I by more than 30%, whereas MoAb 11-7 was less efficient (reduced by only 6%). These results suggest that both E1 and E2 are major allergenic epitopes but that E3 is only a minor one. Further characterization of E1 and E2 reveals that they are labile in alkaline but resistant to acid and sodium periodate treatments. Moreover, E1 is heat-labile, but guanidine- and urea-sensitive, whereas E2 is not. Both E1 and E2 lost their antigenicity after reduction and alkylation.

Conclusions: Results of the present study provide important information on the physicochemical properties of major allergenic epitopes on *Cyn d* I, which may be useful for future development of therapeutic peptides for patients allergic to Bermuda grass pollen. (*J ALLERGY CLIN IMMUNOL* 1993;91:1035-41.)

Key words: Epitope, allergenic epitope, *Cyn d* I, allergen, Bermuda grass pollen, monoclonal antibody

Bermuda grass *Cynodon dactylon* pollen (BGP) is an important allergen, and *Cyn d* I is the major allergenic component of BGP,¹⁻⁵ and has been shown to have different isomeric forms with molecular weights that range from 29 to 35 kd.

To identify and characterize the allergens of BGP, we have generated a panel of 16 monoclonal antibodies (MoAbs) and have classified them according

Abbreviations used

<i>Cyn d</i> I:	Group I allergen of <i>Cynodon dactylon</i> pollen
BGP:	Bermuda grass pollen
IgE:	Immunoglobulin E
MoAb:	Monoclonal antibody
RAST:	Radioallergosorbent test
RIA:	Radioimmunoassay
PBS:	Phosphate-buffered saline
PBS-BSA:	Bovine serum albumin in phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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to the antigens that they recognize.¹ Among the eight categories of anti-BGP MoAbs (categories I to VIII), a group of four anti-BGP MoAbs (category VI) was found to react specifically with *Cyn d* I.¹ In a radioimmunoprecipitation test the antigen recognized by these four MoAbs had a molecular weight of 35 kd and consists of at least four isomeric forms. In addition,

a modified RAST disclosed that this antigen has the strongest IgE binding capacity and the highest IgE binding frequency (91%) among the eight antigens that were tested. These four MoAbs are designated as anti-*Cyn d I* MoAbs.

It is common knowledge that the use of pure allergen for hyposensitization can avoid collateral immune response to other irrelevant components. Accordingly, clinical allergists have noticed that the use of specific major allergenic epitope for immunotherapy could efficiently prevent further development of allergy.⁶ Therefore epitope analysis has not only academic but also clinical significance.

In this study we report the use of anti-*Cyn d I* MoAbs to identify and characterize their associated epitopes. In addition to grouping them and determining whether they are major allergic epitopes, we have characterized some of their physicochemical properties. This study increases our understanding of *Cyn d I* epitopes and provides important information on the development and future application of therapeutic peptides for BGP-allergic patients.

MATERIALS AND METHODS

Anti-*Cyn d I* MoAbs

Four anti-*Cyn d I* MoAbs (MoAbs 4-37, 11-7, 10-7, and 1-61), were obtained as previously described.¹ Briefly, hybridomas were generated by fusion of NS-1 myeloma cells with splenic cells of BALB/c mice that were immunized with BGP crude extracts. Sixteen MoAbs secreted by different hybridomas were grouped according to the antigens they recognized. With the use of a radioimmunoprecipitation assay, the molecular weights and isoelectric points of the antigen molecules were illustrated on two-dimensional gels.¹ In addition, the allergenicity of these antigen molecules was studied with a modified RAST. All four MoAbs were found to react with the 35 kd major allergen of BGP.

After cloning and subcloning, the hybridomas were injected into BALB/c mice, and ascites were collected. MoAbs were purified with a protein-A column. After dialysis, the protein content of MoAbs was determined by the Lowry method.⁶ Purified MoAbs were lyophilized and stored at 1 mg/tube until analysis.

Cyn d I

The major allergen of BGP was purified with a MoAb affinity column (manuscript submitted) with a previously described method.⁷ Briefly, MoAb 4-37 was coupled to CNBr-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) at a concentration of 4 mg/ml according to the manufacturer's instruction and packed into an affinity column. The BGP extracts were loaded onto the MoAb 4-37 immunosorbent column at a flow rate of 5 ml/hr. Unbound antigens were washed off with phosphate buffered saline (PBS), and the column was then eluted with 0.1 mol/L citric acid (pH 3). The pH of the eluents was adjusted to 7.0 with 1 mol/L Tris buffer and dialyzed against distilled

water. The protein contents were determined by the Lowry method.⁸ After purification, *Cyn d I* showed a single 34 kd band or two bands with molecular weights of 32 kd and 29 kd on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sequencing of *Cyn d I* from polyvinylidene difluoride membrane was performed according to the method of Chang et al.⁹ The N terminal sequence of *Cyn d I* is Ala-Met-Gly-Asp-Lys-Pro-Gly-Pro-X-Ile-Thr-Ala-Thr/Lys-Tyr-Gly-Asp-Lys-Trp-Leu-Asp-Ala-Lys-Ala-Thr-Phe-Tyr-Gly-Asp-Asp-X (x for uncertain) (manuscript submitted).

Iodination

Purified MoAbs were iodinated by the chloramine-T method as described previously.¹⁰

Inhibition of binding RIA

Epitopes recognized by the MoAbs were grouped by an inhibition of binding radioimmunoassay (RIA).¹⁰ Briefly, *Cyn d I* was coated onto wells of polyvinyl plates at a concentration of 100 ng/well. The unsaturated sites were blocked with PBS that contained 0.3% bovine serum albumin (PBS-BSA). Different dilutions of anti-*Cyn d I* MoAbs (10-fold serial dilution, diluted from 1 mg of purified MoAbs) were then added to the wells. MoAb 9-13, which reacts with a minor allergen of BGP,¹ was used as MoAb control, and PBS-BSA was used as negative control. After 1 hour of incubation, unbound MoAbs were washed off with PBS. Iodine-125-labeled anti-*Cyn d I* MoAbs (1×10^5 cpm/well) were added and reacted for 1 hour at room temperature. After thorough washing with PBS-BSA, the radioactivity in each well was determined by a gamma counter (LKB, 1271 RIAgamma, Turku, Finland). The percentage of inhibition of binding was calculated as the counts per minute of control minus counts per minute of test over the counts per minute of control.

RAST inhibition

To analyze whether the epitopes recognized by MoAbs could be recognized by human IgEs, a RAST inhibition test was performed.⁹ Briefly, different dilutions of MoAbs were added to *Cyn d I*-coated wells. PBS-BSA was used as background control, and a nonspecific MoAb (MoAb 9-13) was used as MoAb control. After reaction at 37° C for 1 hour, unbound MoAbs were washed off with PBS, and sera of BGP-allergic patients (1:5 diluted) were added and incubated for another hour at room temperature. Iodinated anti-human IgE MoAb E17-58¹¹ was added to the treated wells at a concentration of 5×10^4 cpm/well. After incubation at room temperature for 1 hour, wells were washed thoroughly with PBS-BSA, and radioactivity was detected with a gamma counter. Percentage of RAST inhibition was calculated as the counts per minute of control minus counts per minute of test over the counts per minute of control.

Thermal treatment

The effect of heating on the antigenicity of E1 and E2 was determined by a competitive RIA. BGP extracts (milligrams per milliliter) were incubated at 22° C (control),

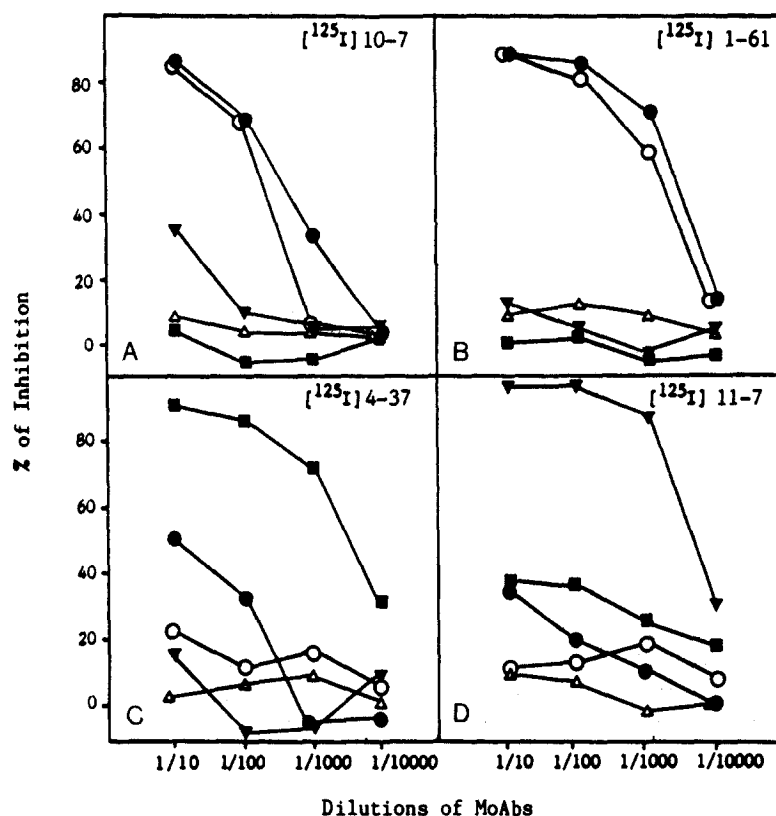


FIG. 1. Inhibition of binding of ^{125}I -labeled MoAb to *Cyn d I* by unlabeled MoAbs (●, 10-7; ■, 4-37; ▼, 11-7; ○, 1-61). MoAb 9-13 was used as control (Δ). Data are averages of triplicates from a representative experiment with standard deviation less than 5%.

37° C, 56° C, 70° C, or 100° C for 5 minutes to 2 hours, and PBS was used as control. The treated BGP extracts were then mixed with anti-*Cyn d I* MoAbs (1:1, vol/vol) and loaded onto plates coated with *Cyn d I* (100 ng/well). After incubation at 37° C for 1 hour, the bound MoAbs were detected with ^{125}I -labeled goat anti-mouse immunoglobulin (5×10^4 cpm/well). The wells were then washed thoroughly with PBS-BSA. The radioactivity in each well was determined with a gamma counter. Percentage of inhibition of binding was calculated as the counts per minute of control minus counts per minute of test over the counts per minute of control.

Periodate oxidation

To determine whether carbohydrate moiety was present on the epitopes, the modified method of Woodward et al.¹² was used. BGP extracts were coated onto wells of polyvinyl plates, then rinsed with 50 mmol/L sodium acetate buffer (pH 4.5), and then exposed to varying concentrations of sodium periodate (0 to 0.1 mmol/L) in 50 mmol/L sodium acetate buffer for 1 hour at room temperature in the dark. At the end of incubation, the wells were washed with sodium acetate buffer and incubated with 1% (wt/vol) glycine at room temperature for another hour. After washing in PBS-BSA, MoAbs were added and incubated for 1 hour. MoAb 9-13, which reacts with an epitope sensitive to the treatment of sodium periodate,¹⁰ was used as the positive control. The

bound MoAbs were detected as described above, and the percentage of binding was calculated as the counts per minute of test over the counts per minute of control.

Effects of pH and other chemical treatments

The effect of pH on the epitope stability was assessed by treating the BGP extracts with 20 mmol/L HCl (pH 2.0) or 20 mmol/L NaOH (pH 12.0) at room temperature for 2 hours.¹³ After neutralization and dialysis against PBS, the samples were used for the competitive RIA. MoAbs were mixed with these treated BGP extracts (1:1, vol/vol) or PBS (control). Aliquots (50 μl) of the mixtures were added to *Cyn d I* coated wells (250 ng/well) and incubated at 37° C for 1 hour. After washing, the MoAbs bound to the solid-phase *Cyn d I* were detected as described above, and the percentage of inhibition of binding was calculated as the counts per minute of control minus counts per minute of test over the counts per minute of control.

To determine the effect of conformational changes on antigenicity of E1 and E2, 250 μg BGP extracts were incubated in either 6 mol/L guanidine-HCl or 6 mol/L urea in 0.25 mol/L Tris-HCl/1 mmol/L ethylenediaminetetraacetic acid buffer, pH 8.5, for 4 hours. They were then dialyzed overnight against PBS and used for competition assay as described above. For reduction and alkylation, 250 μg of BGP extracts was incubated in 6 mol/L guanidine-HCl, 0.25 mol/L Tris-HCl/1 mmol/L EDTA/0.5% 2-mer-

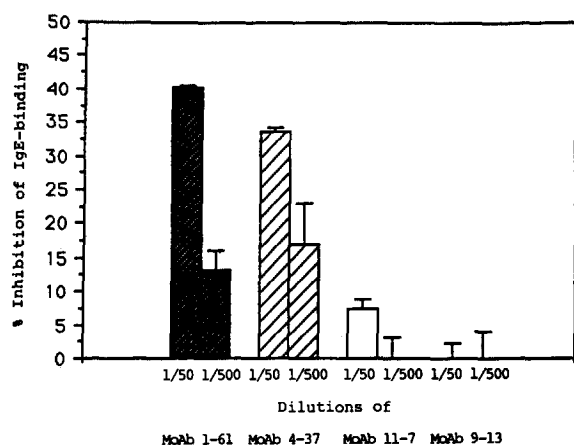


FIG. 2. Inhibition of IgE binding to *Cyn d I* by MoAbs. *Cyn d I* was coated onto 96-wells plates at a concentration of 100 ng/well. Different dilutions of MoAbs (1:50 and 1:500) were used as blocking antibodies to inhibit the binding of IgEs from BGP-allergic patients to *Cyn d I*. Data are means of triplicates from a representative experiment.

captoethanol, pH 8.5, for 2 hours in the dark and sparged intermittently with nitrogen. Reduced cystine residues were pyridyl ethylated by reacting with 4-vinyl pyridine (final concentration, 4%) for 2 hours.

Immunoblotting

Immunoblotting was performed as previously described.¹⁰ *Cyn d I* was dissolved in Laemmli sample buffer and heated at 100° C for 2 to 30 minutes. The samples were separated on an 11% SDS-PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane (Millipore Corp., Bedford, Mass.) and treated with 3% (wt/vol) gelatin. Membrane strips were then reacted with MoAbs 4-37 and 1-61 at room temperature for 1 hour. After washing with Tris buffer (1 mol/L, pH 7.2), strips were incubated with peroxidase-labeled goat anti-mouse IgG (1/1500 dilution, Bio-Rad Laboratories, Chemical Div., Richmond, Calif.) for 1 hour. The blots were then thoroughly washed, and bound peroxidase was detected by addition of a freshly prepared solution of 0.05% (wt/vol) 4-chloro-1-naphthol (Bio-Rad Laboratories) and 0.015% H₂O₂ in Tris buffer. Finally, blots were washed in distilled water and air dried.

RESULTS

Epitope specificity

Fig. 1 shows the results of the inhibition-binding RIA. MoAb 10-7 inhibited the binding of itself to *Cyn d I* in a dose-dependent manner. In addition, the binding of MoAb 10-7 to *Cyn d I* was inhibited by MoAb 1-61 to a similar degree but not by MoAb 4-37 or MoAb 11-7. No significant inhibition was observed for the control MoAb 9-13 (Fig. 1, A). Similarly, the binding of MoAb 1-61 to *Cyn d I* was inhibited by MoAb 1-61 and MoAb 10-7 in a dose-dependent manner but not by MoAb 4-37 and MoAb 11-7 (Fig.

1, B). These results indicate that MoAb 10-7 and MoAb 1-61 recognize the same (or a very similar) epitope. This epitope is designated as E1.

In Fig. 1, C and D, binding of MoAb 4-37 and MoAb 11-7 to *Cyn d I* was inhibited by themselves but not by other MoAbs. It suggests that the epitopes recognized by MoAb 4-37 and MoAb 11-7 are distinct and different from E1. They are designated as E2 and E3, respectively.

IgE binding capacity

Fig. 2 shows the results of inhibition of *Cyn d I* RAST by MoAbs. Sera of eight BGP-allergic patients were pooled for the RAST. The control MoAb 9-13 did not inhibit the binding of IgE to *Cyn d I*. Conversely, MoAb 1-61 inhibited 40.2% of the binding of human IgEs at a dilution of 1:50 and was capable of causing a 13.1% inhibition of IgE binding at a dilution of 1:500. Similar results were found with MoAb 4-37; a 33.4% and 16.9% inhibitions of IgE binding were noticed at dilutions of 1:50 and 1:500, respectively. This suggests that E1 and E2 are both recognized by human IgEs. In contrast, MoAb 11-7 inhibited only 6.2% of binding of human IgEs to *Cyn d I* at a dilution of 1:50, and no inhibition of IgE binding was observed at a dilution of 1:500. In a separate experiment, when a mixture of MoAbs 4-37, 1-61, and 11-7 (diluted 1:50) was used for inhibition of IgE binding to *Cyn d I*, an inhibition ratio of 59.8% was observed.

Because only E1 and E2 are dominant epitopes, E3 is excluded from the rest of the study.

Thermal stability

Table I shows the results of a competitive RIA. After heating at 56° C for 1 hour, E1, which was recognized by MoAb 1-61, lost its competitive ability; the inhibition of binding capacity decreased from 40.8% to 5.4%. On the other hand, the antigenicity of E2 remained intact under the same conditions, but the inhibition ratio decreased from 37.1% to 2.4% after treatment at 70° C for 1 hour.

In an immunoblotting analysis the antigenicity of E2 remained after boiling for 30 minutes (Fig. 3).

Periodate oxidation

Table II shows the effect of periodate oxidation on epitope recognition by different MoAbs. MoAb 9-13 was used as the positive control. As shown, treatment of BGP extracts with sodium periodate (0.1 mmol/L) significantly interfered with the antigenicity of the epitope recognized by MoAb 9-13; the binding ratio decreased from 97.8% to 10.6%. In contrast, the binding of MoAb 1-61 and MoAb 11-7 to their antigenic

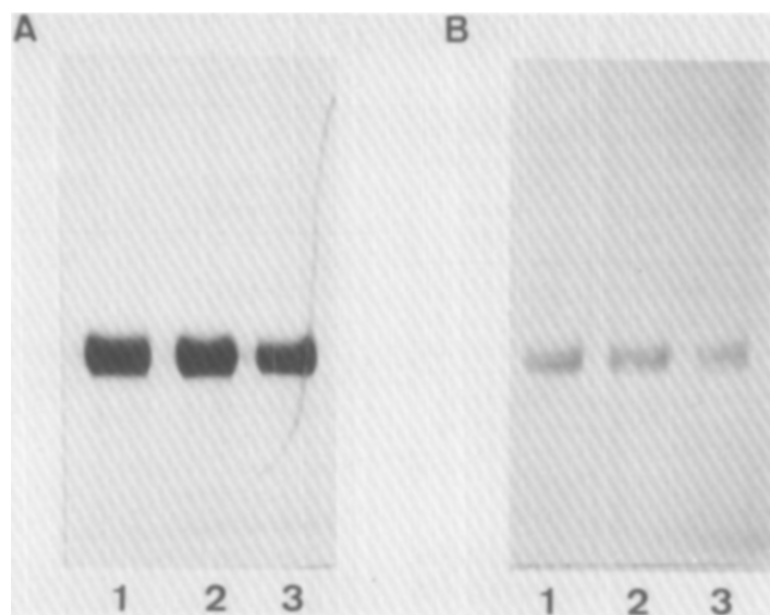


FIG. 3. The effect of thermal denaturation on the antigenicity of E2, also studied by an immunoblot assay. **A**, An 11% SDS-PAGE was performed to illustrate *Cyn d* I (2 μ g/lane), which had been dissolved in Laemmli sample buffer and boiled for 2, 10, and 30 minutes, respectively. **B**, The gel containing *Cyn d* I of **A** was transferred onto a slice of nitrocellulose membrane and reacted with MoAb 4-37 followed by enzyme labeled goat anti-mouse IgG and its substrate, respectively.

determinants was unaffected, and the binding ratios were 90.6% and 93.8%, respectively. This indicates that the antigenicity of E1 and E2 is resistant to sodium periodate treatment.

Effects of pH and other chemical treatments

In Table III the control (BGP treated with PBS) was capable of a 34.0% binding inhibition by MoAb 1-61 to E1 on *Cyn d* I. The percentage of inhibition of binding for E2 was also 34.0%. After treatment of BGP with HCl (pH 2), the competitive ability of the antigen components for both E1 and E2 was unaffected (31.0% and 38.0%). Nevertheless, the competitive capacity of E1 decreased to 7.6% and that of E2 to 2.6% after treatment with NaOH (pH 12).

Different effects on the antigenicity of E1 and E2 were observed after treatments with guanidine and urea. The antigenicity of E1 was significantly diminished; the guanidine treatment decreased the competitive ability of E1 from 34.0% to 3.2%, whereas urea treatment reduced it to 6.2%. In contrast, the same treatments had no effect on the antigenicity of E2; the inhibition of binding ratios was 32.0% for guanidine treatment and 25.5% for urea treatment.

Reduction and alkylation of BGP extracts completely destroyed the antigenicity of E1 and E2, and their competitive abilities decreased to -7.1% and -3.7%, respectively.

TABLE I. The effect of thermal denaturation on the antigenicity of E1 and E2 studied by a competitive RIA

Treatment	Percent inhibition of binding	
	MoAb 1-61	MoAb 4-37
37° C, 1 hr	40.8 $\pm$ 2.9	37.1 $\pm$ 0.5
56° C, 1 hr	5.4 $\pm$ 2.5	32.2 $\pm$ 0.9
70° C, 1 hr	5.9 $\pm$ 4.1	2.4 $\pm$ 0.9
100° C, 5 min	2.6 $\pm$ 3.7	0.5 $\pm$ 1.4

BGP extracts (1 mg/ml) were treated at different temperatures and mixed with Anti-*cyn d* I MoAbs (1:1). Aliquots of 50 μ l of the mixtures were then added onto *Cyn d* I coated wells (200 ng/well) and incubated at 37° C for 1 hour. MoAbs bound to the solid phase *Cyn d* I were then detected with ¹²⁵I-labeled goat-anti-mouse-Ig.

DISCUSSION

Previously, we have demonstrated that our anti-*Cyn d* I MoAbs do not cross-react with four other grass pollens (canary, cane, annual blue, and corn).¹ This indicates that E1, E2, and E3 are present only on *Cyn d* I but not on other group I allergens. The specificity has been confirmed by recent data that all of these anti-*Cyn d* I MoAbs do not cross-react with 10 other grass pollens, including ryegrass pollen and Kentucky blue grass pollen (manuscript submitted).

TABLE II. The effect of periodate oxidation on the antigenicity of E1 and E2 studied by RIA

Concentration of sodium periodate (mmol/L)	Percentage of binding of MoAbs to BGP extracts treated with sodium periodate		
	MoAb 9-13	MoAb 1-61	MoAb 4-37
0.001	97.8 ± 7.1	105.4 ± 5.1	119.2 ± 13.3
0.01	77.0 ± 3.7	112.4 ± 7.7	111.7 ± 0.5
0.1	10.6 ± 2.4	90.6 ± 14.0	93.8 ± 13.4

The binding of different MoAbs to BGP extracts that had been treated with different concentrations of sodium periodate was detected by an RIA with ¹²⁵I-labeled goat-anti-mouse IgG (5 × 10⁴ cpm/well).

Matthiesen et al.⁵ also generated four MoAbs against *Cyn d* I, but they are all cross-reactive with other grass pollens. Our anti-*Cyn d* I MoAbs were obtained by immunizing BALB/c mice with BGP crude extracts and antigenic components they recognized had been characterized by immunoblotting, radioimmunoprecipitation, and modified RAST. Conversely, Matthiesen et al.⁵ purified *Cyn d* I by concanavalin A chromatography and ion-exchange chromatography and used the material directly for BALB/c immunization of mice and generation of anti-*Cyn d* I MoAbs. The preparation of MoAbs and purity of *Cyn d* I may lead to the difference observed between the anti-*Cyn d* I MoAbs obtained by us and those by Matthiesen et al.⁵

In addition to mapping these three *Cyn d* I-specific epitopes (Fig. 1), we show that these three epitopes are all human IgE binding epitopes (Fig. 2). With the use of MoAbs in combination with inhibition of human IgE binding assay, epitopes on major allergens of many pollens such as *Amb a* I, *Amb a* III, *Lol p* I, *Lol p* IV, *Dac g* I, and *Poa p* I have been mapped.¹⁴⁻¹⁹ The major allergen of ragweed pollen, *Amb a* I, was found to consist of a few distinct epitopes,¹⁴ and an MoAb inhibited more than 40% of the binding of IgEs to *Amb a* I. On the other hand, a lot of nonoverlapping epitopes (at least five) were found on *Lol p* I, so that a 10% of inhibition of IgE binding was considered to be a significant result.¹⁵ The finding that about 30% of RAST inhibition was observed when MoAbs 4-37 and 1-61 were added (1:50) and only 6% of IgE binding was inhibited by MoAb 11-7 (1:50) (Fig. 2) strongly suggests that E1 and E2, but not E3, are the major allergenic epitopes of *Cyn d* I. The finding that E1 and E2 are present only on *Cyn d* I but not on other grass pollens correlates well with the observation by Schmachter et al.²⁰ that sera

TABLE III. Effects of chemical treatments on the antigenicity of E1 and E2 studied by competitive RIA

Treatment of competitors	Percent inhibition of binding	
	MoAB 1-61 to E1	MoAb 4-37 to E2
PBS	34.0 ± 2.6	34.0 ± 3.2
Change in pH		
pH 2	31.0 ± 1.5	38.0 ± 2.0
pH 12	7.6 ± 0.9	2.6 ± 1.2
Conformational change		
Guanidine (6 mol/L)	3.2 ± 1.7	32.0 ± 1.2
Urea (6 mol/L)	6.2 ± 2.1	25.5 ± 1.7
Reduction and alkylation		
2-mercaptoethanol, guanidine and 4-vinyl pyridine	-7.1 ± 3.5	-3.7 ± 1.9

BGP extracts that had been treated in different ways were mixed with anti-*Cyn d* I MoAbs (1:1). Aliquots of 50 µl of the mixtures were then added onto *Cyn d* I coated wells (200 ng/well) and incubated at 37° C for 1 hour. MoAbs bound to solid-phase *Cyn d* I were then detected with ¹²⁵I-labeled goat-anti-mouse-Ig.

of BGP-allergic subjects are minimally absorbed by other grass pollens.

Major allergenic epitopes, E1 and E2, have different physicochemical properties (Tables I to III). The resistance of E1 and E2 to sodium periodate treatment (Table II) suggests that the carbohydrate vicinal hydroxyl group is not an integral part of their antigenic determinant.¹²

Changes in three-dimensional configuration induced by denaturation or chemical modification of a protein may result in the complete loss of antigenicity of conformational epitopes. During characterization of epitopes on *Amb a* I, it was found that any modification would destroy its allergenicity, and MoAbs raised against denatured *Amb a* I did not bind the native form of this allergen.¹¹ It thus appears that most of the epitopes on *Amb a* I are conformational rather than sequential. The conformational stability of epitopes on *Der p* I and *Der p* II had been studied by Lombardero et al.,¹³ and epitopes were distinguished on the basis of their susceptibility to physicochemical denaturation. The methods of Lombardero et al.¹³ were applied to the present study (Table III). The finding that antigenicity of E1 and E2 is resistant to the acidic treatment is reasonable because our *Cyn d* I, which was purified with affinity chromatography by citric acid (pH 3), can still react with the MoAbs. The observation that these epitopes are sensitive to alkaline treatment suggests that irreversible denaturation or

modification took place. This may be caused by destruction of amino acid side chain(s), such as deamidation²¹ or rearrangement of hydrogen bonds during alkaline treatment and subsequent neutralization, which folds the protein into a conformation that differs from the native state. Moreover, treatments with guanidine and urea had different effects on the antigenicity of E1 and E2 (Table III). The results correlate with our immunoblotting data, which demonstrate that the antigenicity of E2 remained unchanged after isoelectric focusing and SDS-PAGE (manuscript submitted), whereas that of E1 was diminished after similar treatment (unpublished results). In addition, E1 is heat-labile (Table I), whereas E2 is heat-stable (Fig. 3). The reason that E2 lost its antigenicity after incubation at 70° C for 1 hour is unknown (Table I). It may relate to the masking of E2 after heat-induced conformational changes. The different susceptibility of E1 and E2 to various physical and chemical denaturations clearly suggests that E1 is highly conformation-dependent and that E2 is not.

A reduced competitive ability of E2 after reduction and alkylation of *Cyn d* I (Table III) may imply that E2 is not strictly sequential. However, possibilities such as the presence of cystine as part of a sequential epitope or the change that results in the internalization of sequential sites after reduction still exist.¹³

To ensure that E2 is a sequential epitope, studies are now in progress to investigate B cell epitopes on *Cyn d* I at the molecular level in order to determine whether a synthetic peptide that represents the sequence of E2 can inhibit the binding of human IgEs to *Cyn d* I.

In conclusion, we have identified and characterized three *Cyn d* I specific allergenic epitopes with the use of a panel of specific MoAbs. Among them, E1 and E2 are major ones. E1 is highly conformation-dependent, and E2 appears to be rather sequential. This information may be useful for the development of therapeutic peptides for the treatment of BGP-allergic patients.

REFERENCES

1. Chang ZN, Tsai LC, Chi CW, et al. Analysis of allergenic components of Bermuda grass pollen by monoclonal antibodies. *Allergy* 1991;46:520-8.
2. Shen HAD, Wang SR, Tang RB, Chang ZN, Su SN, Han SH. Identification of allergens and antigens of Bermuda grass (*Cynodon dactylon*) pollen by immunoblot analysis. *Clin Allergy* 1988;18:401-9.
3. Ford SA, Baldo BA. Identification of Bermuda grass (*Cynodon dactylon*)—pollen allergens by electroblotting. *J ALLERGY CLIN IMMUNOL* 1987;79:711-20.
4. Matthiesen F, Schumacher MJ, Lowenstein H. An immunoelectrophoretic analysis of the allergens of *Cynodon dactylon* (Bermuda grass) pollen. *J ALLERGY CLIN IMMUNOL* 1989;83:1124-34.
5. Matthiesen F, Schumacher MJ, Lowenstein H. Characterization of the major allergen of *Cynodon dactylon* pollen, *Cyn d* I. *J ALLERGY CLIN IMMUNOL* 1991;88:763-74.
6. Lowenstein H. New and improved vaccines for the 1990s. *Clin Exp Allergy* 1991;21(suppl 1):227-31.
7. Tsai LC, Chen HM, Huang H, Lin JK, Wu WL, Tsai YC. A simple procedure to purify carcinoembryonic antigen. *Asia Pac Commun Biochem* 1987;1:133-7.
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.
9. Chang LH, Hwieh JC, Chen WL, Tam MF. Identification of rat liver glutathione S-transferase Yb subunits by partial N-terminal sequencing after electroblotting of proteins onto a PVDF membrane from an analytical isoelectric focusing gel. *Electrophoresis* 1990;265:16210-5.
10. Chang ZN, Chi CW, Sun HF, Tasi LC, Lee DT, Han SH. A unique human IgE-binding epitope on the Bermuda grass pollen recognized by mouse lambda-type monoclonal antibodies. *Clin Exp Allergy* 1991;21:503-9.
11. Chang ZN, Chang LD, Wang MC, Tsai LC, Ling CY, Chi CW, Han SH. Monoclonal antibodies specific for human IgE and their clinical applications. *Proc Natl Sci Counc Repub China [B]* 1988;12:140-5.
12. Woodward MP, Young WW Jr, Bloodgood RA. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods* 1985;78:143-53.
13. Lombardero M, Heymann PW, Platts-Mills TAE, Fox JW, Chapman MD. Conformational stability of B cell epitopes on group I and group II *Dermatophagoides* spp. allergens. Effect of thermal and chemical denaturation on the binding of murine IgG and human IgE antibodies. *J Immunol* 1990;144:1353-60.
14. Olson JR, Klapper DG. Two major human allergenic sites on ragweed pollen allergen antigen E identified by using monoclonal antibodies. *J Immunol* 1986;136:2109-15.
15. Bose R, Rector ES, Fischer J. Production and characterization of mouse monoclonal antibodies to allergenic epitopes on *Lol p* I (Rye I). *Immunology* 1986;59:309-15.
16. Atassi H, Atassi MZ. Antibody recognition of ragweed allergen Ra 3: localization of the full profile of the continuous antigenic sites by synthetic overlapping peptides representing the entire protein chain. *Eur J Immunol* 1986;16:229-37.
17. Mourad W, Mecheri S, PeLtre G, David E, Hebert J. Study of the epitope structure of purified *Dac g* I and *Lol p* I, the major allergens of *dactylis glomerata* and *Lolium perenne* pollens, using monoclonal antibodies. *J Immunol* 1988;141:3486-91.
18. Jaggi KS, Ekramoddoullah AK, Kisil FT, Dzuba-Fisher JM, Rector ES, Sehon AH. Identification of two distinct allergenic sites on ryegrass-pollen allergen, *Lol p* IV. *J ALLERGY CLIN IMMUNOL* 1989;83:845-52.
19. Lin Z, Ekramoddoullah AKM, Jaggi KS, Dzuba-Fisher J, Rector E, Kisil FT. Mapping of epitopes on *Poa p* I and *Lol p* I allergens with monoclonal antibodies. *Int Arch Allergy Appl Immunol* 1990;91:217-23.
20. Schmacher MJ, Grabowski FJ, Wagner CM. Anti-Bermuda grass RAST binding is minimally inhibited by pollen extracts from ten other grasses. *Ann Allergy* 1985;55:584-7.
21. Wright HT. Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit Rev Biochem Mol Biol* 1991;26:1-52.