

Zo-Nan Chang^a
Ming F. Tam^b
Chia-Chen Liu^a
Chin-Wen Chi^c
Ho-Jen Peng^c
Shou-Hwa Han^a

^a Faculty of Medical Technology,
Research Center for Immunology, and
Institute of Microbiology and Immunology,
National Yang-Ming University;
^b Institute of Molecular Biology,
Academia Sinica,
^c Department of Medical Research and
Education, Veterans General Hospital-
Taipei, Taipei, Taiwan, Republic of China

Using Monoclonal Antibodies to Characterize a Sequential Epitope on the Group I Allergen of Bermuda Grass Pollen

Abstract

Background: Cyn d 1, the group I allergen of Bermuda grass pollen, had been purified and characterized. **Methods:** A sequential B cell epitope on Cyn d 1 was studied with monoclonal antibodies (MoAbs). Cyn d 1 was cleaved by Achromobacter protease I into fragments, and the resulting peptides were fractionated on reversed-phase columns before being reacted with anti-Cyn d 1 MoAbs in a radioimmunoassay. A Cyn d 1 fragment recognized by its MoAb was selected for Edman degradation. A synthetic peptide was constructed according to the determined sequence. **Results:** The epitope on Cyn d 1 recognized by MoAb 18–53 was found to be conformation independent, since its activity was not changed after sodium periodate, guanidine or urea treatment. The enzyme-cleaved fragment containing this epitope was determined to be DVDKPPFDGMTACGNE-PIF which corresponds to the N-terminal 46–64 residues of Cyn d 1. The presence of this sequence in the epitope recognized by MoAb 18–53 was demonstrated by enzyme immunoassay and further confirmed by inhibition of binding enzyme immunoassay with synthetic peptides. Some cross-reactivity with the N-terminal 45–63 residues of Lol p 1 was also found. **Conclusions:** The primary structure of a sequential epitope on Cyn d 1 was determined, and its activity was confirmed with peptides synthesized according to the determined sequence.

Key Words

Sequential epitope
Monoclonal antibody
Group I allergen
Bermuda grass pollen

Introduction

The knowledge of sequential B cell epitopes on allergens is important not only for basic research but also for the development of therapeutic agents. Type I hypersensitivity arises from degranulation of mast cells triggered by cross-linking of high-affinity IgE receptors through the binding of a multivalent allergen to its IgE antibodies. Thus, monovalent synthetic peptides incapable of cross-linking IgE molecules seem to be ideal agents for immunotherapy. Recently, Ball et al. [1] isolated an immunodominant IgE epitope ex-

pressed from the cDNA library of Phl p 1 [1]. This recombinant epitope bound specifically to IgE antibodies from patients allergic to grass pollens without inducing histamine release by basophiles. This successfully demonstrated the potential usage of specific haptens in immunotherapy.

There are a number of approaches to study B cell epitopes. Besides analyzing the antigenicity of randomly expressed allergen fragments in recombinant DNA libraries [2], B cell epitopes can also be predicted using computer programs [3] or determined by analyzing overlapping synthetic peptides identical to the allergen [4]. In addition, anti-

KARGER

E-Mail karger@karger.ch
Fax +41 61 306 12 34
http://www.karger.ch

© 1997 S. Karger AG, Basel
1018–2438/97/1143–0258 \$12.00/0

This article is also accessible online at:
http://BioMedNet.com/karger

Correspondence to: Zo-Nan Chang, Associate Professor
Faculty of Medical Technology
National Yang-Ming University
Taipei, Taiwan 112 (Republic of China)

genic determinants can also be studied using a gene-targeted phage display random epitope library [5].

Cyn d 1, the 32- to 34 kD allergenic component of Bermuda grass (*Cynodon dactylon*) pollen (BGP), has been purified and characterized [6–11]. In the present study, a sequential B cell epitope on Cyn d 1 was studied using a monoclonal antibody (MoAb) cocktail in combination with Cyn d 1 peptides generated by protease cleavage. The cocktail was made of two anti-Cyn d 1 MoAbs, 4–37 and 18–53. MoAb 4–37 was previously generated [8, 12], and MoAb 18–53 was newly generated. The present study disclosed MoAb 18–53 to be against a nonconformational epitope, and reported the characterization of this epitope. The presence of this sequential epitope on Cyn d 1 fragment was confirmed using a synthetic peptide, and this described some possible first steps to attain the hapten-based immunotherapies.

Materials and Methods

Anti-Cyn d 1 MoAbs

Four MoAbs were used in the present study. Among these four MoAbs, three (MoAbs 4–37, 10–7 and 18–53) are against Cyn d 1, and another MoAb 9–13 is against a cross-reactive epitope on a minor allergen of BGP. The generation and characterization of MoAbs 4–37, 10–7, and 9–13 had been previously reported [12], and the newly obtained MoAb 18–53 was also generated using the same hybridoma technique. The MoAbs 4–37 and 10–7 are against different epitopes on Cyn d 1; MoAb 4–37 recognizes a nonconformational epitope whereas MoAb 10–7 reacts with a conformational epitope [8]. MoAb 9–13 is against a carbohydrate-cross-reactive epitope on the 68-, 48-, 38-kD minor allergenic components of BGP [13]. The isotypes of MoAbs 9–13, 4–37, 10–7, and 18–53 are all IgG1k.

Cyn d 1

The 32- to 34-kD major allergen of BGP was purified with MoAb 4–37 immunosorbent affinity column as previously described [7]. Briefly, MoAb 4–37 was coupled to CNBr-activated Sepharose 4B beads at a concentration of 4 mg/ml as suggested by the manufacturer (Pharmacia, Uppsala, Sweden). This was then used to pack an affinity column and the BGP extract was loaded onto this column at a flow rate of 5 ml/h. The column was washed with phosphate-buffered saline (PBS, 150 mM sodium phosphate, pH 7.4, 150 mM NaCl) and the bound antigen was eluted off the column with citric acid (0.1 M, pH 3). After dialysis against distilled water, the eluent was lyophilized and was ready for further study.

Sodium Periodate Treatment

This was performed as previously described by Woodward et al. [14]. Briefly, the 96-well vinyl assay plates (Costar, Cambridge, Mass., USA) were coated with BGP (50 µg/ml, 50 µl/well) in carbonate buffer (50 mM, pH 9.6) overnight at 4°C, and free sites were blocked by 1% bovine serum albumin (BSA) at 37°C for 30 min. After coating, the plates were rinsed with sodium acetate buffer (50 mM, pH 4.5), and exposed to varying concentrations of sodium periodate (0–1 mM) in sodium acetate buffer for 1 h at room temperature in the dark.

At the end of the incubation, the wells were washed with sodium acetate buffer and incubated with 1% glycine at room temperature for another hour, and followed by washing with PBS containing 0.5% BSA. The wells were then subjected to a radioimmunoassay (RIA) as previously described [12]. MoAbs were added to wells and incubated for 1 h. MoAb 9–13 was used as the positive control. Iodine-125-labeled goat anti-mouse Ig (Kirkegaard and Perry Laboratories, Gaithersburg, Md., USA) was then added at a concentration of 1×10^5 count per minute (cpm) per well, and reacted for 1 h at room temperature. This was followed by thorough washing with PBS-BSA. Radioactivity in the wells was determined with a gamma counter (LKB, 1271 RIA gamma, Turku, Finland). The percentage of binding was expressed as the cpm of test over the cpm of non-treated control.

Acid and Alkaline Treatments

This was performed as previously described [8]. Cyn d 1 was treated with 20 mM HCl (pH 2.0) or 20 mM NaOH (pH 12.0) at room temperature for 2 h. After neutralization and dialysis against PBS, the treated samples were used for a competitive RIA. Supernatant of the hybridoma was mixed with the treated Cyn d 1 (1:1, vol/vol) or PBS (control). Aliquots (50 µl) of the mixture were added onto Cyn d 1 coated wells (250 ng/well) and incubated at 37°C for 1 h. After washing, the bound MoAb was detected with iodine-125-labeled goat anti-mouse Ig. The percentage of inhibition was calculated as the cpm of control minus cpm of test over the cpm of control.

Denaturation Treatments

The effect of conformational changes on epitopes of Cyn d 1 was detected as previously described [8]. Aliquots of 250 µg of Cyn d 1 were incubated with either 6 M guanidine-HCl or 6 M urea in 0.25 M Tris-HCl/1 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.5) for 4 h. It was then dialyzed overnight against PBS and used for a competitive RIA. For reduction and alkylation, 250 µg of Cyn d 1 was incubated with 6 M guanidine-HCl in 0.25 M Tris-HCl containing 1 mM EDTA, 0.5% 2-mercaptoethanol (pH 8.5) for 2 h 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 h.

Enzymatic Digestion

Cyn d 1 (1 mg/ml) was digested with endopeptidase Achromobacter protease I (Lys-C) (Wako, Osaka, Japan) at 37°C for 18 h, in 100 mM of $(\text{NH}_4)_2\text{CO}_3$, pH 9.5, with an enzyme-to-substrate ratio of 1:100 as described by Lin et al. [15]. The resulting peptides were separated with high-performance liquid chromatography (HPLC).

HPLC Fractionation of Cyn d 1 Digests

HPLC analysis was performed on an ABI model 140B solvent delivery system (Applied Biosystems Inc., Foster City, Calif., USA) equipped with a model 1000S diode array detector as previously described [7]. Solvent A was 0.08% (vol/vol) trifluoroacetic acid in water and solvent B was 80% acetonitrile in water containing 0.05% trifluoroacetic acid. Lys-C-digested Cyn d 1 (100 µg) was loaded onto an Aquapore (Pierce, Rockford, Ill., USA) C₈ reversed-phase column (0.46 cm i.d. $\times$ 22.0 cm) that was equilibrated with 15% buffer B. Elution was carried out at a flow rate of 0.6 ml/min. A linear gradient of 0.5% per minute of buffer B was started 5 min after sample injection. The elution profile was monitored at 220 nm. Fractions were collected according to UV absorption and lyophilized. Samples used in RIA were resuspended in PBS before coating onto wells of RIA plates.

Fragments obtained from the C₈ reversed-phase column were further separated on a Vydac C₁₈ reversed-phase column (HP Analytical, Hesperia, Calif., USA) (0.21 cm i.d. × 25.0 cm). The lyophilized samples were dissolved in solvent A, and loaded onto the C₁₈ column that was equilibrated with 20% solvent B. A linear gradient of 0.25% per minute of solvent B was started 5 min after sample injection.

Amino Acid Sequence Determination

The HPLC fractionated fragments that have demonstrated the binding capacity of MoAbs 4–37 and 18–53 were subjected to Edman degradation on an Applied Biosystems Model 470A protein-peptide gas-phase sequencer equipped with an on-line phenylthiohydantoin-amino acid analyzer (ABI model 120 A, Applied Biosystems, Inc.) as previously described [7].

Synthetic Peptides

Peptides were synthesized by Bio Synthesis Laboratories (Levittown, Tex., USA). Peptide ¹AMGDKPGCITATYGDKWLD²⁰ corresponds to the N-terminal 1–20 amino acid residues of Cyn d 1 [7], and peptide ⁴⁵DVDKAPFNGMTGCGNTPIF⁶³ corresponds to the N-terminal 45–63 residues of Lol p 1 [16].

EIA

The synthetic peptides were coated onto the wells of the EIA plates at a concentration of 200 ng/well. MoAbs were added and incubated at 37°C for 1 h. After washing, the peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, Calif., USA) was added to the wells and incubated at 37°C for 1 h. The bound antibody was then detected with the substrate 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid. Absorbance at 405 nm was detected on a Titertek Multiskan instrument (Flow Laboratories, McLean, Va., USA). In the binding inhibition assay, aliquots of MoAb 18–53 (62.5 ng/ml) were mixed (1:1) with different concentrations (0.01–100 µg/ml) of competitors (⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴, Cyn d 1, and ¹AMGDKPGCITATYGDKWLD²⁰) or with PBS (control). The mixtures were incubated at 4°C overnight, and aliquots of 50 µl were added to the Cyn d 1-coated plates (100 ng/well) to perform an EIA. The percentage of inhibition was calculated as the difference in optical density (OD) between control and competitor over the OD of control.

Results

Whether the epitope recognized by MoAb 18–53 is sensitive to sodium periodate treatment was evaluated with a RIA (table 1). MoAb 9–13, that has been shown to be reactive against a carbohydrate epitope of some minor allergens of BGP [12], was used as a positive control; hence BGP was used in the RIA instead of Cyn d 1. As shown, sodium periodate treatment diminished the reaction between MoAb 9–13 and BGP in a dose-dependent manner. However, this treatment did not affect the reaction between MoAb 18–53 and BGP.

Table 2 shows the effect of various chemical treatments on the antigenicity of the epitope recognized by MoAb 18–53. MoAb 4–37, that has been identified as reactive against a nonconformational epitope [8], was used as a control. In a

Table 1. The effect of periodate oxidation on the antigenicity of the epitope on BGP recognized by MoAb 18–53

Concentration of sodium periodate, mmol/l	Binding of MoAbs to BGP extracts, %	
	MoAb 9–13	MoAb 18–53
1	9.7±0.8	93.0±9.3
0.1	10.6±2.4	98.8±6.1
0.01	77.0±3.7	100.7±4.1

Data are the mean ± SD of triplicates from a RIA.

Table 2. Effects of chemical treatments on the antigenicity of the epitope on Cyn d 1 recognized by MoAb 18–53

Treatment	Inhibition of binding to Cyn d 1, %	
	MoAb 4–37	MoAb 18–53
PBS	44.0±3.5	68.6±5.3
Change in pH		
pH 2	48.0±2.8	76.0±6.1
pH 12	4.4±2.9	63.9±8.0
Conformational change		
Guanidine (6 mol/l)	43.1±3.2	56.2±3.3
Urea (6 mol/l)	45.5±5.7	66.9±4.6
Reduction and alkylation		
2-Mercaptoethanol, guanidine and 4-vinyl pyridine	–12.4±3.1	–9.7±0.2

Data are the mean ± SD of triplicates from a competitive RIA.

competitive inhibition assay, the control Cyn d 1 (treated with PBS) inhibited the binding of MoAb 18–53 to Cyn d 1 by 68.6%. Acid, alkaline, guanidine and urea treatments have similar effects on the competitive ability of the epitope recognized by MoAb 18–53. Inhibition ratios of 76.0, 63.9, 56.2 and 66.9%, respectively, were obtained for these treatments. However, the epitope lost its antigenicity when Cyn d 1 was reduced and alkylated. The competitive ability significantly decreased to –9.7%. The control MoAb 4–37 has reactions similar to all the chemical treatments except that its competitive ability was totally lost after treatment with alkaline: the inhibition ratio reduced from 44.0 to 4.4%.

The Lys-C digests of Cyn d 1 were not subjected to reduction and alkylation to avoid interfering with the antigenicity of the epitope. A C₈ reversed-phase column was used to isolate the peptide fragments, and a total of 16 fractions were collected (fig. 1). These fragments were then screened

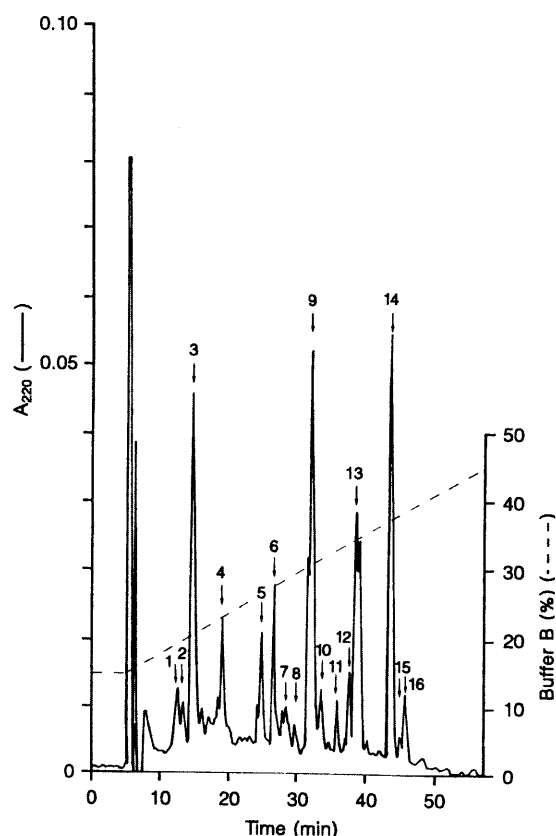


Fig. 1. HPLC profile of the Lys-C-digested Cyn d 1 separated on a C_8 reversed-phase column ($C_8 \times 4.6$ mm). The fractionations were eluted with solvents A and B as described in Methods. A total of 16 fractions were collected at different percentages of solvent B: 18.4, 19.1, 19.9, 22.0, 24.9, 25.9, 26.8, 27.4, 28.7, 29.4, 30.6, 31.5, 32.0, 34.4, 35.4 and 36.7.

with a mixture of MoAbs 4–37 and 18–53 in a RIA. Fraction 9 that eluted off the column with 28.7% solvent B, showed a positive RIA result.

Fraction 9 was further fractionated on a C_{18} column, and three subfractions (C8-9-1, C8-9-2 and C8-9-3) were obtained (fig. 2). Table 3 lists the properties of these three subfractions. The amino acid sequence of these three fragments was determined to be DVIPANW, DVDKPPFDGMTA/GNEPIF, and NYE/IAAY, respectively. We could not pick up any meaningful signal for the 13th residue of C8-9-2 and the fourth residue of C8-9-3 during sequence analysis. The results suggest a cysteine residue or a glycosylation site might occur at these positions. A RIA showed that among these three subfractions, C8-9-2 was the one that most strongly reacted with the anti-Cyn d 1 MoAbs; the binding of the MoAb cocktail to C8-9-2 was 313 cpm, whereas that

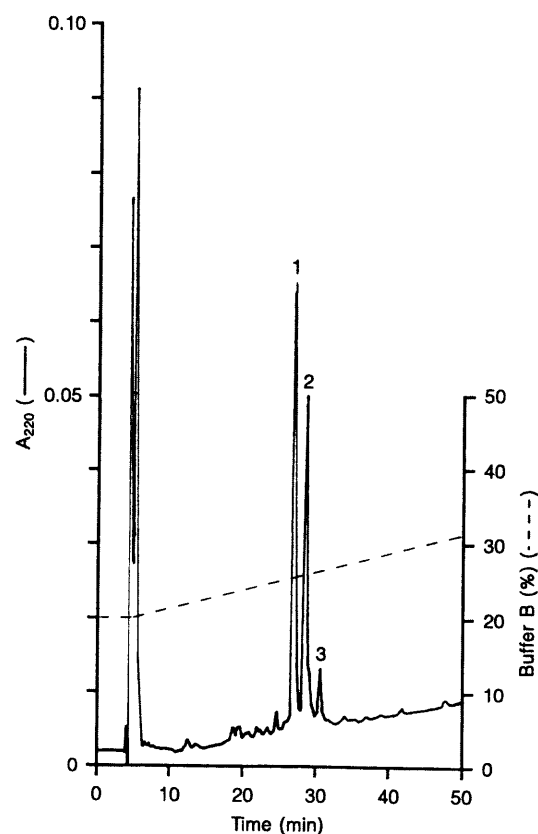


Fig. 2. HPLC profile of the C8-9 fraction rechromatographed on a C_{18} reversed-phase column. The fractionations were eluted with a gradient of solvents A and B as described in Methods.

to the C8-9-1 and C8-9-3 were 120 and 92 cpm. The RIA between the MoAb cocktail and the positive (Cyn d 1) and negative (BSA) controls were 1,035 cpm and 108 cpm, respectively.

Synthetic peptides were constructed according to the amino acid sequences of these three fractions. Cysteine was used on the 13th residue of C8-9-2 and the 4th residue of C8-9-3. Since these three peptides correspond to fragments starting from the N-terminal 226th, 46th, and 97th residues of Cyn d 1 [11], they were marked as ^{226} DVIPANW ^{232} , ^{46} DVDKPPFDGMTACGNEPIF ^{64} , and ^{97} NYECIAAY ^{104} , respectively. Peptide ^{1} AMGDKPGPCITATYGD ^{20} WLD, that represents the first 20 N-terminal amino acids of Cyn d 1 was used as a nonspecific control. Additional controls included the positive control Cyn d 1 and the negative control BSA. These peptides were applied to an EIA to react

Table 3. Analysis of the ninth fraction of Lys-C digested Cyn d 1 fragments

Fraction	RIA with MoAb cocktails cpm	Amino acid sequence
C8-9-1	120	DVIPANW
C8-9-2	313	DVDKPPFDGMTA/GNEPIF
C8-9-3	92	NYE/IAAY

Phenylthiohydantoin-amino acids were not detected in these cycles during Edman degradation.

Table 4. Analysis of the interaction between synthetic peptides and anti-Cyn d 1 MoAbs by an EIA

Antigens	EIA with MoAbs		
	18-53	4-37	10-7
Peptides			
¹ AMGDKPGPCITATYGDKWLD ²⁰	0.245 ±0.012	0.262 ±0.015	0.253 ±0.005
²²⁶ DVIPANW ²³²	0.260 ±0.024	0.239 ±0.013	0.231 ±0.017
⁴⁶ DVDKPPFDGMTACGNEPIF ⁶⁴	1.283 ±0.061	0.223 ±0.016	0.241 ±0.029
⁹⁷ NYECIAAY ¹⁰⁴	0.244 ±0.014	0.258 ±0.027	0.228 ±0.012
Positive control			
Cyn d 1	1.084 ±0.103	1.137 ±0.058	1.119 ±0.066
Negative control			
BSA	0.205 ±0.012	0.237 ±0.014	0.213 ±0.015

Data are the absorbance at OD 405 nm (mean ± SD of triplicates).

Table 5. Analysis of the cross-reactivity between MoAb 18-53 and Lol p 1 specific peptide by an EIA

Peptides	EIA with MoAbs	
	18-53	4-37
Lol p 1 peptide		
⁴⁵ DVDKAPFNGMTGCGNTPIF ⁶³	0.358±0.027	0.253±0.026
Positive control		
⁴⁶ DVDKPPFDGMTACGNEPIF ⁶⁴	0.582±0.048	0.242±0.010
Negative control		
¹ AMGDKPGPCITATYGDKWLD ²⁰	0.181±0.011	0.185±0.023

Data are the absorbance at OD 405 nm (mean ± SD of triplicates).

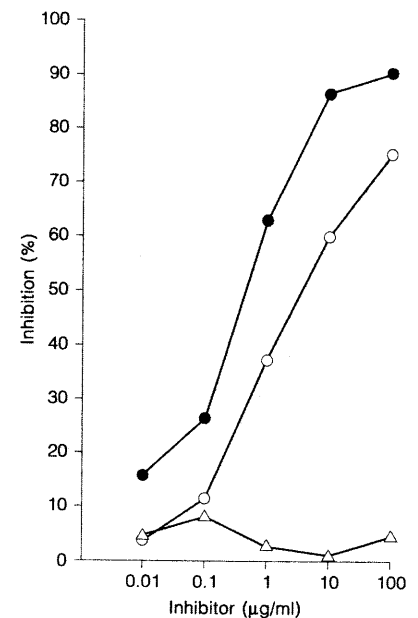


Fig. 3. Inhibition of binding of MoAb 18-53 to Cyn d 1 by peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴. Binding of MoAb 18-53 to the solid phase Cyn d 1 was inhibited by different concentrations of competitors (○ = Cyn d 1; ● = ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴; △ = ¹AMGDKPGPCITATYGDKWLD²⁰). Data are averages of triplicates. The SDs are less than 10%.

with MoAbs 18-53 and 4-37. Another anti-Cyn d 1 MoAb 10-7, that has been identified to be against conformational epitope [8] was used as a negative control. As shown in table 4, MoAb 18-53 reacted with peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ but not with the other three peptides. The reaction between peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ and MoAb 18-53 was specific because it did not react with MoAbs 4-37 and 10-7.

In addition, an inhibitive EIA (fig. 3) showed that the binding between MoAb 18-53 and Cyn d 1 was blocked dose-dependently by the peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴, and Cyn d 1 (positive control), but not by the peptide ¹AMGDKPGPCITATYGDKWLD²⁰ (negative control).

We further tested the cross-reactivity of MoAb 18-53 against Lol p 1, the group 1 allergen of rye grass pollen. A peptide with the sequence DVDKAPFNGMTGCGNTPIF was synthesized according to the cDNA information of Lol p 1 [16], and an EIA was performed (table 5). Peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ was used as the positive control, and peptide ¹AMGDKPGPCITATYGDKWLD²⁰ as

the negative control. As shown, MoAb 18-53 reacted with the Lol-p-1 specific peptide ⁴⁵DVDKAPFNGMTGCGNT-PIF⁶³ (OD: 0.358). However, the reaction between the MoAb and the Lol p 1 specific peptide was weaker (61.5%) than that between MoAb 18-53 and the Cyn d 1 specific peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ (OD: 0.582).

Discussion

It is well known that most epitopes on antigen molecules are conformation dependent. Sequential epitopes determined by the linear amino acid sequence are less common but can be easily reconstructed by peptide synthesis. The complete sequence of Cyn d 1 was unclear when we started the present study, but it has recently been elucidated [11].

First, the nonconformational nature of the epitopes recognized by our anti-Cyn d 1 MoAb 18-53 was clarified by biochemical studies (tables 1, 2). Mild periodate oxidation at acidic pH can cleave carbon bonds between vicinal hydroxyl groups of the carbohydrate moieties of glycoproteins without altering the structure of the polypeptide chain [14]. The finding that sodium periodate treatment did not affect the reaction between MoAb 18-53 and its antigen (table 1) suggests that carbohydrates are not involved in the antigenicity of this determinant; the epitope recognized by MoAb 18-53 appeared to be protein in nature. The findings that treatments of Cyn d 1 with acid, guanidine and urea hardly had any effect on the competitive ability of the elicited epitope (table 2) strongly suggest that this antigenic determinant was conformation independent; changes in the three-dimensional configuration of a protein by denaturants will result in loss of antigenicity of conformational epitopes [17]. This is further supported by our observation that MoAb 18-53 was capable of recognizing its antigen in immunoblots of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional gels (data not shown). In addition, the fact that the antigenicity of the epitope recognized by MoAb 4-37 was lost after treatment of alkaline whereas that of MoAb 18-53 was not clearly indicates that the epitopes recognized by these two MoAbs are different.

Alkylation of a protein usually occurs in reducing the sample in the presence of strong denaturants. Elimination of disulfide bonds upon reduction in the presence of denaturants would facilitate protein unfolding, which consequently leads to loss of antigenicity of conformational epitopes [17]. Similar to the epitope recognized by MoAb 4-37, the antigenicity of the epitope recognized by MoAb 18-53 was also sensitive to reduction and alkylation. The result might give an impression that these two epitopes are

conformation dependent. However, when a cysteine was used for the unidentified signal on residue 13 of C8-9-2, the peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ was capable of reacting with MoAb 18-53 (table 4). This demonstrates that the peptide contains the primary structure of the epitope recognized by MoAb 18-53, and this was further confirmed by a competitive inhibition assay (fig. 3). It is highly possible that cysteine is part of the sequential epitope since addition of bulky alkylating on sulfhydryl groups of cysteine residue(s) might affect the antigenicity of a sequential epitope which contains cysteine.

Besides fraction 9, fractions 11 and 12 also showed a weakly positive RIA with the MoAb cocktails. Edman degradation analysis detected multiple signals starting with DVDKPPF among fractions 11 and 12 (data not shown). These two fractions had been further separated into another seven and five small subfractions, respectively. However, due to the limited amount of samples obtained, they were excluded from the present study.

The phenomenon that three (9, 11 and 12) out of 16 fractions obtained contained peptides starting with DVDKPPF may relate to the fact that Cyn d 1 was digested without reduction and alkylation. It suggests that this sequence is exposed on the surface of the Cyn d 1, easily accessible to protease and antibody recognition. The antigen probably has a rather compact structure. Digestion with protease in the present condition results in partially digested peptides with varying length and hydrophobicity which can be separated on a reversed-phase column.

Using V8 protease, lysylendopeptidase and pepsin to digest the Cry j 1, its immunoreactive fragments had also been detected with MoAbs [18]. In addition, other reagents such as cyanogen bromide and 2-nitro-5-thiocyanobenzoic acid had also been applied for the studies of B cell epitopes with the use of polyclonal antibodies [19, 20]. Peptide chains are cleaved by cyanogen bromide at the methionine, and cleaved by 2-nitro-5-thiocyanobenzoic acid at the cysteine. In the amino acid composition of Cyn d 1, there are 3-5 methionine residues, 7 cysteine residues, and 24-31 lysine residues [6, 7]. Since Lys-C digestion would result in small peptides whose amino acid sequence would be easier to analyze, the other enzymes were not used in the present study. However, the epitope recognized by MoAb 4-37 was not able to be identified. It is highly possible that lysine residue(s) is in its amino acids sequence.

Peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ has 79% identity with the N-terminal amino acid residues 45-63 of Lol p 1, the group I allergen of *Lolium perenne* [16]. Cyn d 1 and Lol p 1 shares high amino acid sequence homology. However, previously we were not able to find cross-react-

tivity between these two allergens since all 4 anti-Cyn d 1 MoAbs we had were specific for Cyn d 1 without cross-reaction to Lol p 1 [7]. In the present study, the finding that MoAb 18-53 cross-reacted weakly with the Lol-p-1-specific peptide ⁴⁵DVDKAPFNGMTGCGNTPIF⁶³ (table 5) suggests that the sequential epitope on Cyn d 1 identified in the present study with the newly obtained MoAb does share cross-reactivity with an epitope on Lol p 1. This agrees with the report of Matthiesen and Lowenstein that some anti-Cyn d 1 MoAbs cross-reacted with Lol p 1 [6].

The IgE-binding capacity of peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ had been tested with pooled sera derived from patients allergic to BGP. However, no positive result was obtained (data not shown). This correlates with the finding of Smith et al. [11]. Recently, they found that only the recombinant Cyn d 1 expressed in the yeast *Pichia pastoris*, but not that expressed in *Escherichia coli* was recognized by IgE from sera of patients allergic to BGP. It sug-

gested that in Cyn d 1, posttranslational modifications (e.g. glycosylation), which occur in eukaryotic cells are necessary for the production of biologically active form of Cyn d 1. This gives a reasonable explanation to why peptide ⁴⁶DVDKPPFDGMTACGNEPIF⁶⁴ has no IgE binding capacity in humans.

Using MoAb, the present study determined the amino acid sequence of a sequential epitope on Cyn d 1, and confirmed its activity with a synthetic peptide. This offers some information for further immunotherapy studies. Since a mouse B cell epitope is present in the defined peptide, this peptide can be used as hapten in animal models to examine the potential of hapten-based immunotherapy.

Acknowledgments

This work was supported by NSC grant 84-2331-B-010-055.

References

- Ball T, Vrtala S, Sperr WR, Valent P, Susani M, Kraft D, Valenta R: Isolation of an immunodominant IgE hapten from an epitope expression cDNA library. *J Biol Chem* 1994;269:28323-28328.
- Ong EK, Knox RB, Singh MB: Mapping of the antigenic and allergenic epitopes of *Lol p* VB using gene fragmentation. *Mol Immunol* 1995;32:295-302.
- Vik H, Steinvag SK, Elsayed S: Studies of the allergenicity of the amino-terminal epitope (Bed v I 23-38) from birch pollen allergen. *Int Arch Allergy Immunol* 1993;101:89-94.
- Elsayed S, Stavseng L: Epitope mapping of region 11-70 of ovalbumin (Gal d 1) using five synthetic peptides. *Int Arch Allergy Immunol* 1994;104:65-71.
- Wang LF, Du Plessis DH, White JR, Hyatt AD, Eaton BT: Use of a gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5. *J Immunol Methods* 1995;178:1-12.
- Matthiesen F, Schumacher MJ, Lowenstein H: Characterization of the major allergen of *Cynodon dactylon* (Bermuda grass) pollen, *Cyn d* I. *J Allergy Clin Immunol* 1991;88:763-774.
- Han SH, Chang ZN, Chi CW, Peng HJ, Liu CC, Tsai JJ, Tam MF: Use of monoclonal antibodies to isolate and characterize *Cyn d* I, the major allergen of Bermuda grass pollen. *J Allergy Clin Immunol* 1993;92:549-558.
- Han SH, Chang ZN, Chang HH, Chi CW, Wang JY, Lin CY: Identification and characterization of epitopes on Cyn d I, the major allergen of Bermuda grass pollen. *J Allergy Clin Immunol* 1993;91:1035-1041.
- Smith PM, Avijoglu A, Ward LR, Simpson RJ, Knox RB, Singh MB: Isolation and characterization of group-I isoallergens from Bermuda grass pollen. *Int Arch Allergy Immunol* 1994;104:57-64.
- Chang ZN, Liu CC, Tam MF, Peng HJ, Tsai JJ, Han SH: Characterization of the isoforms of the group I allergen of *Cynodon dactylon*. *J Allergy Clin Immunol* 1995;95:1206-1214.
- Smith PM, Suphioglu C, Griffith JJ, Theriault K, Knox B, Singh MB: Cloning and expression in yeast *Pichia pastoris* of a biologically active form of Cyn d I, the major allergen of Bermuda grass pollen. *J Allergy Clin Immunol* 1996;98:331-343.
- Chang ZN, Tsai LC, Chi CW, Wang MC, Shen HD, Lee DT, Han SH: Analysis of allergenic components of Bermuda grass pollen by monoclonal antibodies. *Allergy* 1991;46:520-528.
- Chang ZN, Chi CW, Sun HF, Tsai 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;21:503-509.
- Woodward MP, Young WW Jr, Bloodgood RA: Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods* 1985;78:143-153.
- Lin RY, Shen HD, Han SH: Identification and characterization of a 30 kd major allergen from *Parapenaeus fissurus*. *J Allergy Clin Immunol* 1993;92:837-845.
- Perez M, Ishioka GY, Walker LE, Chesnut RW: cDNA cloning and immunological characterization of the rye grass allergen, Lol p I. *J Biol Chem* 1990;265:16210-16215.
- Lombardero M, Heymann PW, Platt-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;144:1353-1360.
- Kawashima T, Tani M, Usui M, Ando S, Kurimoto M, Matuhashi T: Antigenic analysis of sugi basic protein by monoclonal antibodies: II. Detection of immunoreactive fragments in enzyme-cleaved Cry j I. *Int Arch Allergy Immunol* 1992;98:118-125.
- Cottam GP, Wheeler AW, Cook RM, Moran DM: Immunological properties of chemically produced fragments of rye grass pollen extract. *Immunol Letter* 1988;17:345-349.
- Jaggi KS, Ekramoddoullah AKM, Kisil FT: Allergenic fragments of ryegrass (*Lolium perenne*) pollen allergen Lol p IV. *Int Arch Allergy Appl Immunol* 1989;89:342-348.