

Z.N. Chang^a
C.C. Liu^a
H.C. Perng^b
L.C. Tsai^b
S.H. Han^a

^a School of Medical Technology and Institute of Microbiology and Immunology, National Yang-Ming Medical College, and

^b Department of Medical Research, Veterans General Hospital-Taipei, Taipei, Taiwan, Republic of China

A Common Allergenic Epitope of Bermuda Grass Pollen Shared by Other Grass Pollens

Key Words

Common allergenic epitope
Minor allergen
Bermuda grass pollen
Cyn d I
Lol p I

Abstract

The present study disclosed the cross-reactivity between Bermuda grass pollen (BGP) and other grass pollens using monoclonal antibodies (MAbs) and polyclonal antiserum. MAb 9-13, directed against a group of minor allergens of BGP (*Cyn d* Bd68K, 48K, 38K) was found to cross-react with extracts of ten other grass pollens. Immunoblotting assays illustrated that MAb 9-13 cross-reacted with multiple components of most of these pollens, and the major cross-reactive components had molecular weights of 29-36 kD. The cross-reactivity between BGP and *Lol p* I, the group I allergen of rye grass pollen, was further evaluated; *Lol p* I was recognized by MAb 9-13, but not by our MAbs/polyclonal antiserum against *Cyn d* I, the major allergen of BGP. These results suggest that the epitope recognized by MAb 9-13 is a common (C) epitope shared by *Lol p* I and *Cyn d* Bd68K, 48K, 38K, and *Cyn d* I does not share significant antigenicity with *Lol p* I. In a modified radio-allergosorbent test, IgE antibodies in the serum of BGP-allergic patients reacted mildly with C-epitope-bearing components of both BGP and rye grass pollens, and this binding could be blocked specifically by MAb 9-13. This suggests that in addition to an antigenic cross-reaction, the C epitope can also lead to an allergenic cross-reaction.

Bermuda grass (*Cynodon dactylon*) is one of the important grasses causing allergy [5, 21, 29]. It was previously thought that its pollen differs significantly from other grass pollens in both antigenicity and allergenicity [14, 17, 18, 25]. However, Schumacher et al. [25] found that the activity of serum from patients allergic to Bermuda grass pollen (BGP) is still slightly absorbed by other grass pollens.

Recently, we obtained a panel of 16 monoclonal antibodies (MAbs) by fusing NS-1 cells with splenic cells derived from BALB/c mice immunized with crude extracts of BGP [8]. Characterized by radioimmunoprecipitation and two-dimensional gel electrophoresis, the proteins recognized by these MAbs were grouped and designated according to their molecular weights (MWs) as *Cyn d* Bd68K, 48K, 38K, *Cyn d* Bd200K, *Cyn d* Bd67K,

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Dr. Shou-Hwa Han
Institute of Microbiology and Immunology
National Yang-Ming Medical College
Taipei, Taiwan 11217 (Republic of China)

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Cyn d Bd58K, *Cyn d* Bd46K, *Cyn d* Bd35K, *Cyn d* Bd25K, and *Cyn d* Bd12K. Among them, *Cyn d* Bd35K was found to be the major allergen of BGP [8], and has recently been purified by us with MAb affinity chromatography [10].

The 16 anti-BGP MAbs were grouped, according to the proteins they react with, into eight categories. Among them, only six category I anti-BGP MAbs which react with minor allergens of BGP, *Cyn d* Bd68K, 48K, 38K, was found to cross-react with antigens of four different grass pollens (*Poa annua*, *Zea mays*, *Phalaris minor*, *Sorghum vulgare* var. *saccharatum*) in a radioimmunoassay (RIA), while the other seven categories of MAbs (including four anti-*Cyn d* I MAbs) did not [8].

Recently, *Cyn d* I was purified biochemically by Mathiesen et al. [19]. A polyclonal antiserum was raised with this purified *Cyn d* I and it was found to be capable of cross-reacting with a number of grass pollens including *Alopecurus pratensis* (*Alo p*), *Dactylis glomerata* (*Dac g*), *Holcus lanatus* (*Hol l*), *Lolium perenne* (*Lol p*), *Paspalum notatum* (*Pas n*), *Phleum pratense* (*Phl p*), *Phragmites communis* (*Phr c*), *Poa pratensis* (*Poa p*), *Secale cereale* (*Sec c*), and *Sorghum halepense* (*Sor h*).

Finding cross-reactivity among different allergens is important both for identifying their IgE-binding epitopes and for the precise diagnosis and effective treatment of the allergic disease. The following questions need to be clarified: Is the common epitope shared by BGP and other grass pollens present on the major allergen *Cyn d* I or on allergens *Cyn d* Bd68K, 48K, 38K? What is the distribution of this common epitope among other grass pollens? Does this common epitope also present on the group I allergens? Will it induce allergenic cross-reaction in addition to antigenic cross-reaction?

This study reports our findings that *Cyn d* Bd68K, 48K, 38K share the C epitope with ten other grass pollens, as well as the group I allergen of rye grass pollen. However, this C epitope is not present on *Cyn d* I, and no cross-reactivity between *Cyn d* I and *Lol p* I was observed using polyclonal anti-*Cyn d* I antiserum.

Methods

Pollen Extracts

Defatted pollens were obtained from Allergon (Uppsala, Sweden) and extracted with 0.15 M phosphate-buffered saline (PBS, pH 8.0) as previously described [8]. The extracts were dialyzed against distilled water and filtered through 0.45- μ m filters (Sartorius, Hayward, Calif., USA), and their protein content was determined by the dye-binding method of Bradford [4] with bovine serum albumin (BSA) as standard. They were then lyophilized and kept at 4°C.

Besides *Cyn d*, *Anthoxanthum odoratum* (*Ant o*), *Dac g*, *Festuca elatior* (*Fes e*), *Hol l*, *Lol p*, *Pas n*, *Phalaris canariensis* (*Pha c*), *Phl p*, *Poa p*, *Sor h*, and *Ambrosia elatior* (*Amb e*) were also prepared with the method described above.

Cyn d I, the major allergen of BGP, was purified from crude extracts of BGP with an immunosorbent made of anti-*Cyn d* I MAb 4-37 [10]. *Lol p* I, the major allergen of rye grass pollens, was obtained from the National Institute of Allergy and Infectious Disease (Bethesda, Md., USA).

Anti-BGP MAbs

These MAbs were obtained by fusing splenic cells from BGP-immunized BALB/c mice and NS-1 myeloma cells as previously described [8]. MAbs 9-13 [IgG1] and 26-11 [IgG2b] which had been demonstrated to react against the same epitope on *Cyn d* Bd68K, 48K, 38K [7] were used in this study. Anti-*Cyn d* I MAb 4-37 (IgG1), which reacts with *Cyn d* I, the 35-kD major allergen of BGP [8], was included as a control.

Polyclonal Anti-*Cyn d* I Antiserum

After purification by affinity chromatography, *Cyn d* I underwent HPLC on an Applied Biosystems (Foster, Calif., USA) model 140B solvent delivery system equipped with a model 1000 S diode array detector. Protein separation was carried out on a 5 μ M Vydac C₁₈ reverse-phase column (HP Analytical, Calif., USA). Solvent A was 0.08% (v/v) trifluoroacetic acid in water and solvent B was 80% acetonitrile in water (v/v) containing 0.05% (v/v) trifluoroacetic acid. The *Cyn d* I which had undergone HPLC separation was then used to immunize BALB/c mice for induction of polyclonal antiserum. Briefly, mice were immunized by intraperitoneal injection of 50 μ g of *Cyn d* I emulsified with complete Freund's adjuvant (Difco, Detroit, Mich., USA). They were boosted with 25 μ g of *Cyn d* I in incomplete Freund's adjuvant (Difco) three times at 2-week intervals.

Allergic Serum

Serum was collected at the Allergy Clinic, Veterans General Hospital-Taichung (Taiwan, Republic of China) from allergic patients with a positive skin test to BGP. They were further selected on the basis of their reactivity to BGP and cross-reaction with extracts of *P. annua* and *Z. mays* by a radioallergosorbent test (RAST) [8]. Serum from individuals with a negative response in the skin test was used as a negative control.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously [7]. Briefly, lyophilized pollen extracts were dissolved in Tris buffer containing 2% SDS and 5% 2-mercaptoethanol, and extracted at 100°C for 3 min. Proteins were then separated in 10% SDS-PAGE as described by Laemmli [13]. Low-MW standards (BioRad, Richmond, Calif., USA) used included rabbit muscle phosphorylase b (97 kD), bovine serum albumin (66 kD), ovalbumin (43 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21 kD), and hen egg white lysozyme (14 kD). Two SDS-PAGE gels were run at the same time. Following electrophoresis, one was silver-stained [20], while another one was electrophoretically transferred to nitrocellulose papers (BS 85, 0.45 μ m; Schleicher & Schuell, Dassel, FRG). The nitrocellulose membranes were then blocked with 3% gelatin in Tris buffer and incubated with supernatants of tested MAbs or with dilu-

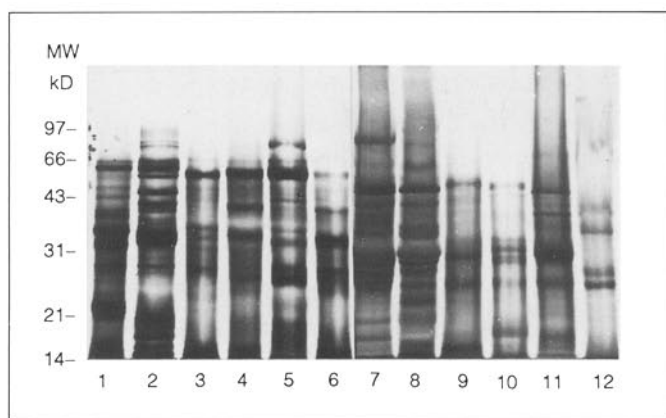


Fig. 1. Protein components in different grass pollen extracts separated by SDS-PAGE. Lanes 1–12 are *Ant o*, *Cyn d*, *Dac g*, *Fes e*, *Hol 1*, *Lol p*, *Pas n*, *Pha c*, *Phl p*, *Poa p*, *Sor h*, and *Amb e*, respectively.

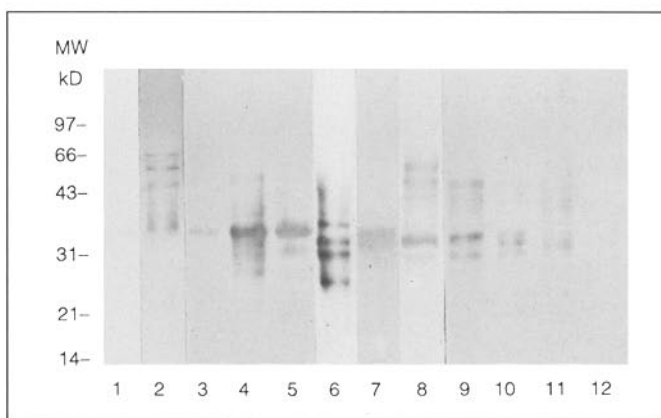


Fig. 2. Immunoblot profiles of C-epitope-bearing components of the pollens illustrated in figure 1. After SDS-PAGE, they were transferred onto slices of nitrocellulose membrane and reacted with MAb 9–13 in an immunoblot assay.

tions of polyclonal antiserum (1:2,500 diluted) at room temperature with gentle shaking for 1 h. After washing with PBS containing 0.05% Tween 20, the membranes were incubated with 1:1,500 diluted peroxidase-labelled goat anti-mouse IgG (Bio-Rad) for another hour. The membranes were then thoroughly washed, and incubated with a freshly prepared solution of 0.05% (w/v) 4-chloro-1-naphthol (Bio-Rad) and 0.015% H_2O_2 in Tris buffer for 30 min. The stained blots were then washed in distilled water and air-dried. Meanwhile, the silver-stained gel was swollen in water to the same size as the transferred gel, and then dried.

Modified RAST

This was performed as described previously [8]. In brief, MAb 26-11 (1 μ g/50 μ l/well) was coated onto polyvinyl plates. The rye pollen extract (5 μ g/well) was added to MAb-coated wells and incubated at 4°C for 18 h. Bermuda pollen and weed pollen extracts at the same concentration were added to MAb-coated wells as positive and negative controls, respectively. After several washings with PBS containing 3% BSA, 50 μ l aliquots of serum from BGP-allergic patients were then added and incubated for 4 h at room temperature. The ^{125}I -labelled anti-IgE MAb E17-58 (5×10^4 cpm/well) [6] was then added and incubated for another hour. The plate was washed again thoroughly with PBS-BSA. Finally, the binding of IgE to the rye pollen antigen recognized by MAb 9-13 was determined with a γ counter (1272 Riagamma, LKB, Turku, Finland).

In the inhibition-of-IgE-binding assay, different dilutions of MAb 9-13 or MAb 4-37 (5-fold diluted from Ab, 0.1 mg/ml) were used as inhibitors to react with molecules of rye grass which were bound indirectly to polyvinyl plates through the MAb in the modified RAST. After washing off the unbound blocking MAb, allergic serum was added and reacted for another hour. The specific IgE binding was detected by ^{125}I -labelled anti-IgE MAb, and the radioactivity was determined with a γ counter after washing.

Results

The crude extracts of 11 species of grass pollens (*Ant o*, *Cyn d*, *Dac g*, *Fes e*, *Hol 1*, *Lol p*, *Pas n*, *Pha c*, *Phl p*, *Poa p*, *Sor h*) and a weed pollen (*Amb e*) were separated on SDS-PAGE and are illustrated in figure 1. As shown, many proteins were found in every pollen extract.

These pollen extracts, after SDS-PAGE fractionation, were transferred onto nitrocellulose membranes and reacted with anti-*Cyn d* Bd68K, 48K, 38K MAb 9-13 and anti-*Cyn d* I MAb 4-37. Except for BGP, their reactions with MAb 4-37 were all negative, and so the results are not shown. The profiles of proteins reacting with MAb 9-13 are shown in figure 2; MAb 9-13 reacted with none of the proteins in the control ragweed extracts (lane 12) but with some C-epitope-bearing components in extracts of all tested grass pollens (lanes 1–11). Except for *Ant o* and *Dac g*, (lanes 1 and 3), the other tested grass pollens contain multiple C-epitope-bearing components. The reactive bands of BGP had approximate MWs of 68, 52, 48 and 38 kD. The MWs of the major reactive bands in other grass pollens (lanes 1, 3–11) were around 29–36 kD, similar to the MWs of the group I allergens of grass pollens.

To evaluate whether the group I allergens of BGP and rye grass pollens also bear the C epitope, these two major allergens were separated on SDS-PAGE (fig. 3, lanes 1 and 2), transferred onto nitrocellulose membranes and then reacted with MAb 9-13 (lanes 3 and 4). An anti-*Cyn d* I MAb 4-37 was used as a control (lanes 5 and 6). In addition, the cross-reactivity between *Cyn d* I and *Lol p* I was further clarified with anti-*Cyn d* I antibodies pooled

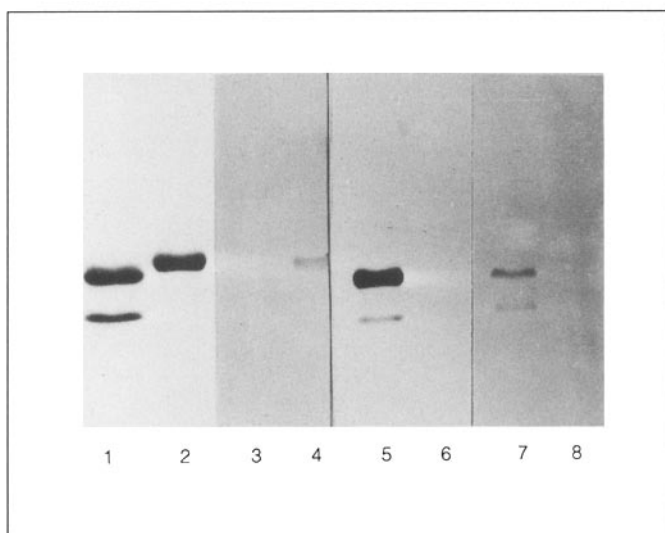


Fig. 3. Reactions of MAbs 9-13, 4-37 and polyclonal anti-*Cyn d* I antiserum with *Cyn d* and *Lol p* I. In an immunoblot assay, *Cyn d* I and *Lol p* I (lanes 1 and 2, respectively) were first separated on 11% SDS-PAGE, then transferred onto a nitrocellulose membrane and reacted with MAb 9-13 (lanes 3 and 4), MAb 4-37 (lanes 5 and 6) or with polyclonal anti-*Cyn d* I antiserum (lanes 7 and 8).

from three immunized BALB/c mice (lanes 7 and 8). As illustrated, MAb 9-13 reacted with *Lol p* I but not *Cyn d* I, while MAb 4-37 reacted only with *Cyn d* I. Pooled mouse anti-*Cyn d* I antiserum (1:2,500 diluted) also reacted only with *Cyn d* I but not with *Lol p* I (lanes 7 and 8).

In the modified RAST, serum from BGP-allergic patients was used to study the allergenic cross-reaction of IgE to rye grass pollen. C-epitope-bearing components of rye grass pollen recognized by category I anti-BGP MAb were bound indirectly to wells of polyvinyl plates and reacted with tested serum. As shown in table 1, serum from four BGP-allergic patients all contained IgE Abs which reacted with the bound C-epitope-bearing molecules of rye grass pollen. No significant IgE binding was observed in the negative pollen control and the normal serum control. Among the four allergic patients, the serum which had the highest IgE binding to BGP also showed the highest IgE binding to rye grass pollen.

Table 2 shows further that the binding of IgEs to the C-epitope-bearing components of rye grass pollen could be inhibited specifically by MAb 9-13 but not by nonspecific anti-*Cyn d* I MAb 4-37. The inhibition was dose dependent, and a high inhibition ratio (57–85%) was observed when the inhibitory Ab was at a dilution of 0.1 mg/ml.

Discussion

Previously, the C-epitope-bearing components of BGP recognized by the six group I anti-BGP MAbs were found to have MWs of 68, 48, and 38 kD in a radioimmunoprecipitation assay [8]. In this assay system, where the preformed ^{125}I -BGP-MAb immune complex was used for SDS-PAGE, it is hard to distinguish whether all three components contain the reactive epitope, or whether they are subunits of an aggregated molecule, among which only one (or two) subunit contains this epitope. This problem was overcome in the present study by using the immunoblot assay which presents the SDS-PAGE-separated proteins in a reduced form and the C-epitope-bearing molecules can be clearly illustrated. The finding that additional components were also capable of reacting with MAb 9-13 in addition to proteins with MWs of 68, 48, and 38 kD (fig. 2), can probably be explained by the fact that immunoblotting is an indirect immunoassay and the reaction between a MAb and a component bearing a limited number of specific epitopes is enhanced after the addition of the second tracing Ab.

In addition to BGP, multiple components of ten other common grass pollens also contain the C epitope (fig. 2). This cross-reactivity may relate to the carbohydrate nature of the C epitope. As previously reported, the C epitope recognized by the category I MAbs was heat stable and very sensitive to treatment by sodium periodate [7]. Recently, different components of *Parietaria judaica* pollen were also shown to share cross-reactivity with carbohydrate moieties [24].

In addition, the C epitope was also present on the group I allergen of rye grass pollen (fig. 3). Using MAbs, many studies have been performed to explore common allergenic components of grass pollens [9, 12, 23, 26, 27]. Kahn and Marsh [12] first reported that anti-*Lol p* I MAbs cross-reacted with BGP to a certain degree in a RIA assay, but the component(s) bearing this cross-reactive epitope was not studied. Using immunoblotting, Esch and Klapper [9] reported that the group I allergens of meadow fescue, June grass, sweet vernal grass, redtop grass and perennial rye grass pollens had the same antigenic determinant, but BGP was not included in their study. Similar results were also reported by Standring et al. [27], and BGP was included in their study. However, the immunoblotting profiles were not shown. These studies suggest that the major (group I) allergens of many grass pollens share a common epitope(s). This is further supported by the finding of Lin et al. [16] that *Poa p* I could be purified from extracts of Kentucky bluegrass pollen with anti-*Lol p*

Table 1. RAST to show the cross-reaction between IgE antibodies in serum from BGP-allergic patients and C-epitope-bearing components in rye grass pollen extracts

Serum	Rye	BGP	Ragweed
A ₁	904 ± 151	847 ± 29	31 ± 11
A ₂	281 ± 28	302 ± 30	17 ± 9
A ₃	358 ± 37	320 ± 16	53 ± 39
A ₄	363 ± 72	305 ± 14	18 ± 9
N ₁	19 ± 7	23 ± 18	24 ± 14
N ₂	12 ± 5	14 ± 14	15 ± 7

In a modified RAST, antigenic components of rye grass pollen bearing the C epitope were bound indirectly to polyvinyl plates by MAb 26-11, and then reacted with serum from BGP-allergic patients. The specific IgE binding was detected by ¹²⁵I-labelled anti-IgE MAbs. BGP and weed pollen served as positive and negative controls, respectively. Data are the mean ± SD of triplicates. A = Allergic; N = normal.

Table 2. Inhibition by MAbs of the binding of IgEs from serum of BGP allergics to C-epitope-bearing components of rye grass pollens

Allergic serum	Dilutions of inhibitory ascites	Inhibition, %	
		MAb 9-13	MAb 4-37
Patient A	1/10	56.6 ± 15.4	14.0 ± 3.4
	1/50	45.3 ± 14.9	2.8 ± 7.1
	1/250	22.6 ± 8.5	0.4 ± 3.6
Patient B	1/10	85.0 ± 6.6	6.8 ± 9.2
	1/50	41.5 ± 15.0	13.6 ± 8.6
	1/250	5.4 ± 9.4	6.7 ± 6.9

In a modified RAST inhibition test, C-epitope-bearing components of rye grass pollens were bound indirectly to polyvinyl plates by MAb 26-11. They were then first reacted with ascites containing anti-BGP MAbs (9-13 or 4-37) at 37°C for 1 h and subsequently reacted with serum of BGP-allergic patients. The specific IgE bound was detected by ¹²⁵I-labelled anti-IgE MAbs. Data are the mean ± SD of triplicates.

I MAb. However, it remains unclear which component of BGP shares the common epitope with group I allergens of other grasses.

The finding that MAb 9-13 cross-reacted with *Lol p I* but not *Cyn d I* (fig. 3) suggests that *Cyn d* Bd68K, 48K, 38K share the C epitope with *Lol p I*; however, this C epitope is absent on *Cyn d I* (fig. 3, lane 3). Furthermore, the finding that pooled anti-*Cyn d I* antiserum did not cross-react with *Lol p I* (fig. 3, lane 8) indicates that no dominant antigenic determinants are shared by *Cyn d I* and *Lol p I*. The use of pooled antiserum here is to avoid unsuccessful sensitization of a single bleeding source. Similar data have also been obtained using the rabbit antiserum (data not shown). We have previously shown that none of our four anti-*Cyn d I* MAbs cross-reacted with the ten species of grasses tested with RIA [10]. The use of polyclonal antiserum here took into consideration the possible existence of some common epitopes which may not be recognized by our available MAbs.

Cyn d I is the major allergen of BGP whereas *Cyn d* Bd68K, 48K, 38K are not [8]. The finding that only *Cyn d* Bd68K, 48K, 38K shared antigenicity with *Lol p I* correlated well with the finding of Standring et al. [27] that anti-*Lol p I* MAbs only cross-reacted mildly with BGP.

The observation that *Cyn d I* did not share dominant antigenic determinants with other grass pollens correlates with previous findings of others that anti-grass polyclonal antiserum was only minimally absorbed by extracts of BGP [14, 17, 18], and also gives a reasonable explanation as to why the antigenicity of BGP had been previously considered to be different from that of other grass pollens. However, this is in contrast to the finding of Matthiesen et al. [19]; their anti-*Cyn d I* MAbs and polyclonal antiserum cross-reacted with ten other grass pollens. The reasons for these different results in cross-reactivity remain unclear. In our preparation, *Cyn d I* was purified by affinity chromatography, then subjected to reverse-phase HPLC before being used to generate polyclonal antiserum. Matthiesen et al. [19] used the *Cyn d I* purified by Con-A Sepharose and ion exchange chromatography directly for immunization.

The mild reaction between IgEs of BGP-allergic serum with C-epitope-bearing components of rye grass pollens in table 1 suggests this may be a cross-reaction. The following factors should be considered. First, rye grass pollen has not been found in Taiwan [11]. The possibility that Taiwanese have been sensitized to rye grass pollens is low. In addition, the C epitope was also found to be present on

Lol p I, the major allergen of rye grass pollens (fig. 3). If patients in table 1 were really allergic to rye grass pollens, their IgEs should have reacted strongly with C-epitope-bearing components of rye grass pollens.

The weak IgE binding capacity of C-epitope-bearing components in table 1 may relate to the carbohydrate nature of the C epitope. Since removing sugars did not significantly reduce the binding property of various allergens to human IgEs, the carbohydrate has been considered to be a weak allergen [24, 28]. Previously, Aalberse [1] also found IgE antibodies reacting with periodate-sensitive cross-reactive carbohydrate determinants among a large number of unrelated allergens (food and pollens). In addition, it was suggested that the presence of IgE antibodies to an allergen does not imply that the patient has ever been exposed to that allergen [2].

In addition to the C epitope, other common epitopes may also lead to the cross-reaction observed in table 1. This possibility is minimized by the observation that the inhibition of IgE binding was specific and the inhibition ratio was high (table 2). The percentage of inhibition of IgE binding seems to relate to the number of epitopes present on allergens [3, 7]. At least five distinct allergenic epitopes were identified on *Lol p I*, and the ratio of the

inhibition of IgE binding by MAbs was as low as 10% [3]. Among epitopes on *Lol p I*, four distinct ones were identified as being shared with *Dac g I* [22] and *Poa p I* [15]. This may explain why these group I allergens show strong cross-reactions. *Lol p I* shares multiple epitopes with group I allergens of other grasses, but only shares the C epitope with BGP. This explains why IgE specific for rye grass pollen was not significantly absorbed by BGP in RAST inhibition assays [14, 18], and correlates well with the previous finding of Schumacher et al. [25] that anti-BGP RAST binding is only slightly inhibited by pollen extracts from ten other grasses.

In conclusion, the present study provides information showing that BGP does share common antigenicity and allergenicity with other grass pollens. However, this common C epitope is present on *Cyn d Bd68K*, 48K, 38K rather than on the major allergenic component *Cyn d I*.

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