

A unique human IgE-binding epitope on the Bermuda grass pollen recognized by mouse lambda-type monoclonal antibodies

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Summary

A group of six mouse monoclonal antibodies (MoAbs) with the unusual lambda-type light chain were generated by fusion of NS-1 cells with splenic cells derived from BALB/c mice immunized with crude extracts of Bermuda grass pollen (BGP). Four of them were IgG1, one was IgG2b, and one was IgG3. Binding inhibition assay showed that they recognized the same (or very similar) epitope. Using sera from BGP-allergic patients, it was found that the specific binding between the IgE antibodies and the MoAb 26-11-fixed antigen could be blocked by MoAb 26-11 itself and another MoAb 9-13 in a dose-dependent manner. It appears that the epitope recognized by the lambda-type MoAbs is a human IgE-binding antigenic determinant. Further physico-chemical analyses showed that this epitope was stable under heat but sensitive to treatments of sodium periodate and proteinase K. Results from these studies indicate that this unique epitope which leads to the generation of lambda-type MoAbs is part of a glycoprotein.

Clinical and Experimental Allergy, Vol. 21, pp. 503–509. Submitted 15 October 1990; revised 15 February 1991; accepted 20 February 1991.

Introduction

Bermuda grass (*Cynodon dactylon*) pollen (BGP) is an important allergenic substance in many areas of the world [1–3]. Its extract contains a variety of protein component and most of them are allergenic [4–6].

Due to technical difficulties, the progress in purification and characterization of major allergens of BGP has been limited. In a previous study, in order to analyse and purify the allergenic components of BGP, anti-BGP MoAbs were generated in two separate fusions and categorized according to the antigenic components they reacted with in immunoblot (unpublished results). Among eight different categories of mouse anti-BGP MoAbs obtained, there was an interesting group of six MoAbs. Four of them were found to be IgG1, one was IgG2b, and one was IgG3. These MoAbs were different from the other groups in that their light chains were all of the rare lambda type; in addition, they cross-reacted with four other grass pollens tested; cane (*Sorghum vulgare* var. *saccharatum*),

corn (*Zea mays*), canary (*Phalaris minor*) and annual blue (*Poa annua*).

It is well known that immunoglobulins with kappa light chains usually outnumber those with lambda light chains by about 20:1 in BALB/c mice. MoAbs with lambda light chains have rarely been reported, and have never been found in the field of allergy. Here we examined the epitope specificity of these lambda-type anti-BGP MoAbs. In addition, the physico-chemical properties of this unique epitope were also probed with heat, sodium periodate and proteinase K treatments.

Materials and methods

Pollen extracts

Defatted BGP was obtained from International Biologicals (Piedmont, OK) and extracted with 0.15 M phosphate-buffered saline (PBS, pH 8.0) at a ratio of 1:10 (g/ml) as previously described [5]. The extract was dialysed against distilled water. The protein content of the crude extract was determined by the method of Lowry *et al.* [7] with bovine serum albumin as a reference control. Protein recovered accounted for 3.0% of the dry pollen. The lyophilized material was stored at -20°C .

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Allergic sera

Sera were collected at the Allergy Clinic, Veterans General Hospital Taichung (Taiwan, R.O.C.) from allergic patients with a positive intradermal skin test to the BGP extract (supplied by Taiwan Allergy Centre, Taipei) as described previously [8], using histamine (0.1 mg/ml) and PBS as positive and negative controls, respectively. The collected sera were further selected on the basis of their strong reactivity in the radioallergosorbent test as described by Ekramoddoullah *et al.* [9] with a slight modification that BGP extracts were coated onto wells of 96-well polyvinyl plate (Costar, Cambridge, MA) at 1 µg/well.

MoAbs

Anti-BGP MoAbs. Splenic cells of BALB/c mice immunized with crude extracts of BGP were fused with NS-1 myeloma cells and cultivated in HAT medium as described by Rector *et al.* [10]. Supernatants of the surviving hybridomas were screened for specific antibody production by RIA and immunoblotting as described previously [5,11]. Hybridomas with positive reactivity were cloned by limiting dilution. Seventeen MoAbs were obtained in two separate fusions (unpublished results). They were grouped into eight different categories (I to VIII) according to their immunoblot profile, and the antigenic components they recognized were reconfirmed by radioimmunoprecipitation test. Light chains of six category I anti-BGP MoAbs were found to be all of lambda type. Four of them were IgG1 (Moabs 9-13, 15-15, 15-3, and 3-12), one was IgG2b (MoAb 26-11) and one was IgG3 (MoAb 16-3). Their ascites were produced and either purified with a protein A column or stored at -70°C. Another anti-BGP MoAb 4-37 with kappa light chains which reacted with a 35K component of BGP was used as control.

Anti-human IgE MoAb. The Anti-Human IgE MoAb E17-58 was generated and characterized in our laboratory as previously reported [12].

Immunoblot

Immunoblot was performed as previously described [5]. Briefly, BGP extracts and molecular weight markers (BioRad, Richmond, CA) were separated in 10% SDS-PAGE according to Laemmli [13]. Separated proteins were electrophoretically transferred to nitrocellulose paper (0.45 µm; BS 85; Schleicher & Schuell, Dassel, Germany), followed by blocking with 3% (w/v) gelatin in 20 mM Tris buffer (pH 7.2). The nitrocellulose membrane was then cut into strips and incubated with MoAbs

(supernatant) at room temperature for 1 hr. After washing with Tris buffer containing 0.05% Tween 20, these strips were incubated with 1/3000 diluted peroxidase-labelled goat anti mouse IgG (BioRad) for 1 hr. The blots were then thoroughly washed, and bound peroxidase was detected by the addition of freshly prepared solution of 0.05% (w/v) 4-chloro-1-naphthol (BioRad) and 0.015% H₂O₂ in Tris buffer. After 30 min, the blots were washed in distilled water and air dried.

Iodination

MoAbs and goat anti-mouse IgG (Kirkegaard and Perry Laboratory, Gaithersburg, MD) were labelled with ¹²⁵I-Na (Amersham, Arlington Heights, IL) by the Chloramine T method [14]. Free ¹²⁵I was removed by gel filtration through a Sephadex G25 column as described by Bolton and Hunter [11].

Inhibition of binding of the radiolabelled MoAb to BGP antigen by unlabelled MoAb

The BGP extract was diluted in 50 mM bicarbonate buffer (pH 9.6) and coated to each well of the polyvinyl RIA plate (Costar) (100 ng/50 µl) at 4°C overnight. The remaining binding sites were blocked by incubation with PBS (0.15 M, pH 7.2) containing 3% (w/v) bovine serum albumin (BSA) at room temperature for at least 30 min. After blocking, inhibitory ascites of different dilutions (starting from 1/10) were added to the plates and incubated at 37°C for 1 hr. PBS containing 0.3% BSA (PBS-BSA) was used as control. The unbound inhibitory MoAbs were removed by extensive washing with PBS-BSA. Afterwards, ¹²⁵I-labelled MoAb 9-13 was added (1 × 10⁵ c.p.m./well) and incubated for 1 hr. The plate was then washed with PBS-BSA, and radioactivity of bound ¹²⁵I-MoAb was determined by a gamma counter (LKB, 1271 RIAGamma, Turku, Finland). Percentage of inhibition of binding was calculated as the net c.p.m. between control and test over the c.p.m. of control.

IgE-binding capacity of the epitope recognized by the lambda-type anti-BGP MoAb

The concentration of MoAb 26-11 used for coating was predetermined. Briefly, the plate was coated with 50 µl of five-fold diluted purified MoAb 26-11 (500–0.8 µg/ml). The diluent used was PBS-BSA. Aliquots of ¹²⁵I-BGP (50 µl, 5 × 10⁴ c.p.m./well) were added and incubated for 1 hr. The plate was washed thoroughly with PBS-BSA and radioactivity in each well was determined. The minimal concentration of MoAb which bound the maximal amount of ¹²⁵I-labelled BGP was selected. Afterwards,

50 μ l of the established optimal concentration of MoAb 26-11 (20 μ g/ml) were coated to 96-well polyvinyl RIA plates. After blocking, each well was then filled with 50 μ l of BGP (5 μ g/well) and the plate was incubated at 4°C for 18 hr. The unbound antigen was removed by several washes with PBS-BSA. Inhibitory ascites of MoAbs 26-11, 9-13 and 4-37 (1/10 diluted or with different dilutions) were added and incubated at room temperature for 1 hr. PBS-BSA was used as control. After washing, 50 μ l of BGP-allergic serum was then added and incubated for 4 hr at room temperature. This was then thoroughly washed with PBS-BSA. Finally, ^{125} I-labelled anti-IgE MoAb was added (5×10^4 c.p.m./well) and the radioactivity was measured after 1 hr of incubation. Percentage of inhibition of IgE binding by anti-BGP MoAb was calculated as the net c.p.m. between the control and the tested samples over c.p.m. of the control.

Physico-chemical treatments of the epitope

Heat treatment. BGP extract (1 mg/ml in PBS) was treated either at 60°C for 30 min, 60 min, or 120 min, or at 100°C for 1 min, 3 min and 11 min, respectively. Afterwards, a competitive RIA was performed. The treated BGP extracts were separately mixed with an equal volume of MoAb 9-13, and an aliquot (50 μ l) of each of the mixtures was added to a RIA plate which had been coated with BGP extracts (100 ng/well). For control, PBS was used instead of BGP extracts. After incubation at room temperature for 1 hr, the plate was washed with PBS-BSA and incubated with ^{125} I-labelled goat anti-mouse IgG (5×10^4 c.p.m./well) for 1 hr. The plate was then washed thoroughly, and radioactivity was detected by a gamma counter. Percentage of inhibition of binding was calculated as the net c.p.m. between control and tested samples over the c.p.m. of control.

Sodium periodate treatment. In order to determine whether carbohydrate moiety was present on the epitope, the method of Woodward *et al.* [15] was used, with some modifications. Briefly, the BGP extract was coated to a PVC plate (100 ng/well) and saturated with 3% BSA. After being rinsed with 50 mM sodium acetate buffer (pH 4.5), the plate was exposed to varying concentrations of sodium periodate (0–1 mM) in 50 mM sodium acetate buffer for 1 hr at room temperature in the dark. It was then washed three times with sodium acetate buffer and incubated with sorbitol (1 M) at room temperature in the dark for 1 hr. This was followed by thoroughly washed with PBS (pH 7.2). After these treatments, MoAb 9-13 and MoAb 4-37 were added and incubated at room temperature for 1 hr. Bound mouse IgG was detected by

^{125} I-labelled goat anti-mouse IgG (5×10^4 c.p.m./50 μ l). After extensive washing with PBS-BSA, the radioactivity in each well was determined by a gamma counter.

Proteinase K treatment. To examine the possible role of proteins play on the epitope, the BGP extract (in 0.5% SDS, 0.1 M Tris, pH 7.5) was digested with either proteinase K (Merck, Darmstadt, Germany) or heat-denatured proteinase K (boiled at 100°C for 10 min) (200 μ g enzyme/mg BGP extract) at 37°C for 2 hr. The enzyme reaction was stopped by boiling at 100°C for 2 min, and immediately cooling to 4°C. The following competitive RIA was carried out at 4°C. Briefly, the treated BGP extracts were mixed with an equal volume of MoAb 9-13. PBS and heat-denatured enzyme (boiled at 100°C for 2 min) were used as control. Aliquots (50 μ l) of each of the mixture were added to wells of BGP coated PVC plates. The bound MoAb 9-13 was then detected by ^{125} I-labelled goat anti-mouse IgG (5×10^4 c.p.m./well).

Results

Immunoblot profiles

The immunoblot profiles of the six lambda-type anti-BGP MoAbs are illustrated in Fig. 1. As shown, these six MoAbs all reacted with the 68, 52, 48 and 38 kD components of BGP.

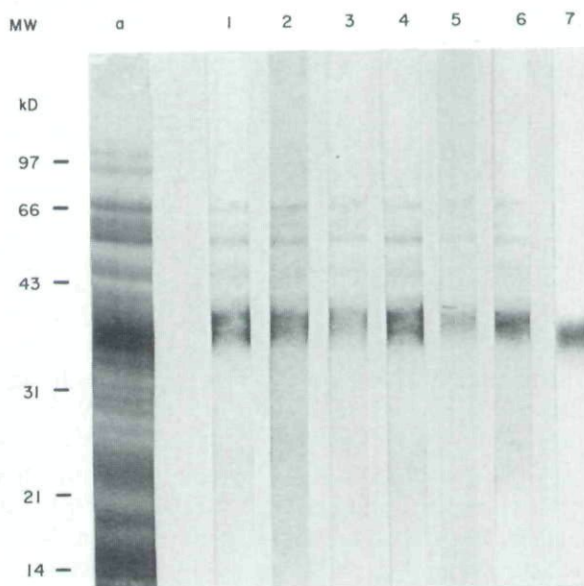


Fig. 1. Immunoblot profiles of MoAbs binding to the components of BGP extracts separated by 10% SDS-PAGE. Lane a, gels stained with Coomassie blue R-250; lane 1–6, lambda-type MoAbs: 1, 9-13; 2, 26-11; 3, 15-3; 4, 15-15; 5, 3-12; and 6, 16-3; lane 7, control MoAb 4-37.

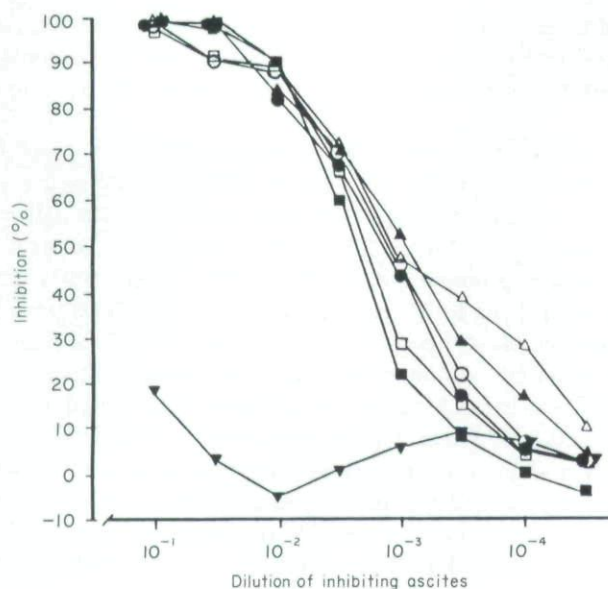


Fig. 2. Inhibition of binding of ^{125}I -MoAb 9-13 to Bermuda grass pollen (BGP) extracts by unlabelled lambda-type MoAbs ($\square$, 9-13; Δ , 26-11; $\circ$, 15-3; $\bullet$, 15-15; $\blacktriangle$, 3-12; $\blacksquare$, 16-3). A kappa-type anti-BGP MoAb ($\blacktriangledown$, 4-37) was used as control. Data are the average of triplicates from one representative experiment. Their s.d. are all less than 10%.

Epitope specificity

In order to examine the epitope specificity of the six lambda-type anti-BGP MoAbs, a binding-inhibition RIA was performed. Figure 2 shows a representative result that all six lambda-type anti-BGP MoAbs inhibited the binding between ^{125}I -labelled MoAb 9-13 and solid-phase BGP antigen effectively in a dose-dependent manner. In contrast, no significant inhibition was found when unrelated anti-BGP MoAb (MoAb 4-37) was used as inhibitor. It indicates that the six lambda-type anti-BGP MoAbs are against the same (or very similar) epitope.

IgE-binding capacity

In a modified radioallergosorbent test, antigen molecules with this epitope were bound indirectly to the PVC plate through MoAb 26-11. In Fig. 3a, all the sera from six BGP-allergic patients contained specific IgE antibodies against the antigen molecules, and the binding of IgE was inhibited by MoAb 26-11. The specificity of the inhibition was reconfirmed in Fig. 3b. As shown, more than 60% of the IgE binding was dose dependently inhibited by MoAb 9-13, but not at all by the control MoAb 4-37.

Physico-chemical treatments

The effect of heat treatment on the epitope recognized by the lambda-type MoAbs is shown in Table 1. After being

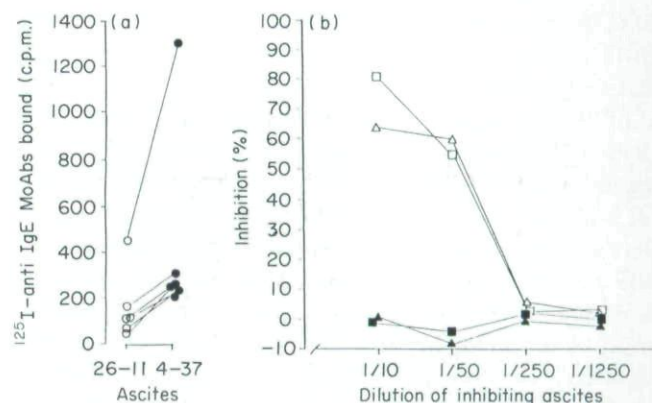


Fig. 3. Inhibition of IgE binding by lambda-type MoAb 9-13. The antigenic components recognized by lambda-type MoAbs were bound indirectly to PVC plates through MoAb 26-11. (a) Binding of specific IgE from sera of six BGP-allergic patients were measured with specific ($\circ$) or nonspecific ($\bullet$) inhibitor ($10\times$ diluted ascites); (b) percentage of inhibition of IgE binding of sera from two BGP-allergic patients ($\square$, Δ) were evaluated with different concentrations of specific (MoAb 9-13, open symbols) and non-specific (MoAb 4-37, solid symbols) inhibitor. Data are the mean of triplicates. The s.d. are all less than 10%.

Table 1. The effect of heat treatment on the epitope recognized by MoAb 9-13

Treatment	Inhibition (%) of binding of MoAb 9-13 to solid-phase antigen
None	63.7 ± 2.0
60°C , 30 min	63.0 ± 2.6
60°C , 60 min	63.8 ± 1.2
60°C , 120 min	64.0 ± 0.7
100°C , 1 min	64.8 ± 2.1
100°C , 3 min	59.8 ± 1.7
100°C , 11 min	5.6 ± 4.5

BGP extracts treated at different temperature for different periods of time were used to compete for MoAb 9-13 with solid-phase BGP extract in a competitive RIA.

Inhibition of binding was calculated as the net c.p.m. between control (PBS was used instead of soluble BGP extract) and tested samples over the c.p.m. of control.

Data are the mean $\pm$ s.d. of three replicates from a representative experiment.

treated at 60°C for 30–120 min, the BGP extract still inhibited the binding of MoAb 9-13 to the solid-phase BGP antigen effectively. Similar results were obtained when the BGP extract was boiled for 3 min. These indicate that the above thermic treatment does not affect

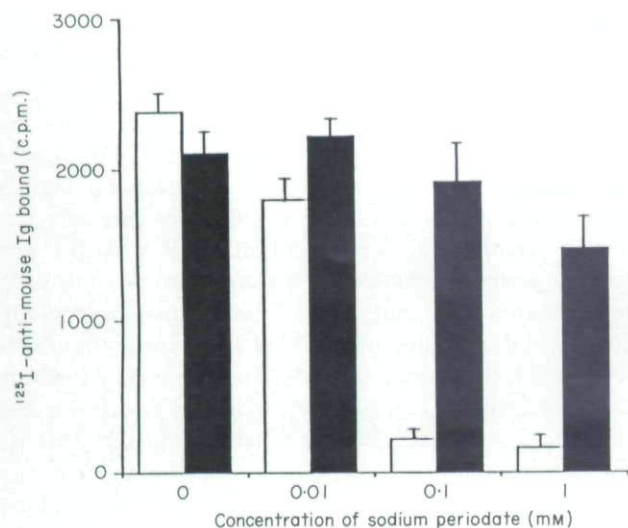


Fig. 4. The effect of periodate oxidation on epitopes recognized by MoAb 9-13 (□) and MoAb 4-37 (■). The binding of MoAbs to BGP extracts which had been treated with different concentrations of sodium periodate was detected by ^{125}I -goat anti-mouse IgG in a RIA. Data are the mean $\pm$ s.d. of three replicates from a representative experiment.

the antigenicity of this epitope. In the control group, boiling at 100°C for 11 min significantly decreased the percentage of binding inhibition to 5.6.

Figure 4 shows the effect of periodate oxidation on the epitope. While sodium periodate was used at a concentration as low as 0.01 mM, there was already a decrease in the binding of MoAb 9-13. When the concentration increased to 0.1 mM, more than 90% of the binding was lost. In contrast, no significant difference in the binding of the control MoAb 4-37 was observed. It is clear that the epitope is highly sensitive to the treatment of sodium periodate.

Table 2 shows the effect of proteinase K on the epitope. BGP extract treated with proteinase K was used to compete for the binding of MoAb 9-13 to the solid-phase BGP antigen. In the presence of PBS and the heat-denatured proteinase K (enzyme control), the total binding was 2642 and 2457 c.p.m., respectively. Untreated BGP extract and BGP extract which had been treated with heat-denatured proteinase K competed effectively for the binding of MoAb 9-13 with total binding decreasing to 1044 and 1119 c.p.m. respectively. However, when BGP antigen treated with proteinase K was used as competitor, the binding of MoAb 9-13 to the solid-phase antigen (2449 c.p.m.) was similar to that of the control. It indicates that this epitope is also sensitive to the treatment of proteinase K.

Table 2. Analysis of the effect of proteinase K on the epitope recognized by MoAb 9-13 using a competitive RIA

Competitor	Binding of ^{125}I -goat-anti-mouse-IgG to MoAb 9-13 (c.p.m.)
PBS	2642 $\pm$ 221
Denatured proteinase K	2457 $\pm$ 162
BGP	1044 $\pm$ 88
BGP treated with proteinase K	2449 $\pm$ 127
BGP treated by denatured proteinase K	1119 $\pm$ 83

BGP extracts were pre-coated to wells of PVC plates. This was then competed for MoAb 9-13 with BGP extracts which had undergone treatment of proteinase K and heat-denatured proteinase K. PBS, heat denatured proteinase K and untreated BGP were used as competitor controls, respectively. The competitive RIA was carried out at 4°C , and the bound MoAb 9-13 was detected by ^{125}I -labelled goat anti-mouse IgG $1(5 \times 10^4)$ c.p.m./well).

Discussion

In addition to having identical immunoblot profile (Fig. 1), the present study (Fig. 2) showed that these six lambda-type MoAbs reacted with the same (or very similar) epitope. This is not unexpected, since they were all of the rare lambda-type MoAb. However, it is conceivable that other antigenic determinants may also exist on the antigen molecules recognized by these lambda-type MoAbs. The reason for the dominance of this particular epitope is not known, although it is possibly related to the screening method used during generation of MoAbs. Since immunoblot analysis was used to select positive hybridoma clones, MoAbs against heat-labile or conformational epitopes may have been excluded [16]. Consequently, the MoAbs generated were against a limited number of epitopes.

Results from this study could not rule out the possibility that kappa-type MoAbs may also be induced by this epitope. One possibility is that this epitope generated mainly lambda-type MoAbs. This is supported by our observation that the epitope could also be recognized by a group of lambda-type MoAbs against extracts of spiny pigweed pollen which also contained a large amount of this epitope (unpublished results). In the process of generation of MoAbs against the extract of spiny pigweed pollen, both kappa-type and lambda-type MoAbs were obtained. However, only lambda-type MoAbs cross-reacted with BGP extract. There were 11 lambda-type anti-spiny pigweed pollen MoAbs obtained, and their

immunoblot profiles were the same as the lambda-type anti-BGP MoAbs.

Despite the low frequency of lambda-type immunoglobulin in normal serum, four antigens have been reported to induce antibodies that contain a high proportion of lambda chains [17–21]. They are: the 4-hydroxy-3-nitrophenylacetyl (NP) group [17], dextrans having an alternating (1-3), (1-6) backbone [18], the 2,4-dinitrophenyl (DNP) group [19,20] and the 5-dimethylaminophthalene-1-sulfonyl (dansyl) group [21]. In the present study, a unique observation was that the sera of BGP-allergic patients contained specific IgE which bound to antigen molecules with the epitope that recognized by these lambda-type anti-BGP MoAbs (Fig. 3). This binding was inhibited specifically by MoAb 26-11 and MoAb 9-13, which suggests that a human IgE-binding epitope was responsible for the generation of mouse MoAbs with lambda light chains. Such an epitope has never been reported before.

Using sera from laboratory animals immunized with crude extracts of allergens, one can identify many antigenic components. However, this did not give a direct guide to which antigens were allergens [22]. In general, molecules recognized by human IgE are usually considered as allergens [22–33]. In order to identify an allergenic epitope, IgE binding inhibition assay is one of the most often-used analyses [23–26].

By using MoAbs in inhibition assay to evaluate whether it can inhibit the reactivity of human IgE with a particular allergen, both allergenic and non-allergenic epitopes have been identified [24–27]. Bose *et al.* [24] identified five distinct allergenic epitopes of *Lol p I* by using 20 MoAbs. Olson and Klapper distinguished two allergenic determinants out of three epitopes on AgE [25]. Ley *et al.* [26] also found two different epitopes which are close to IgE binding sites on antigen molecules in crude extracts of *Dermatophagoides farinae*. Among these studies, percentages of inhibition of IgE binding to allergens by MoAbs varied a lot. A high inhibition (50%) was observed in the study of AgE [25]. In contrast, an inhibition as low as 10% was considered to be significant in studies on *Lol p I* allergen [24]. The difference is related to the number of different epitopes expressed on the allergen [25]. There appear to be only a few kinds of allergenic epitopes on AgE, thus the binding of a MoAb to one kind of epitope caused a high percentage of inhibition. On the other hand, a MoAb which bound to one of several different allergenic determinants on *Lol p I* only led to a low percentage of inhibition. In this study, the percentage of inhibition of IgE binding by MoAb 9-13 was very high (>60%). It indicates that the epitopes recognized by these lambda-type MoAbs are not only allergenic but also dominant.

The nature of this epitope was explored by heating and treatments of sodium periodate as well as proteinase K. It was found that this epitope was heat stable. Heating at 60°C for 120 min or boiling for 3 min did not affect its antigenicity (Table 1). Mild periodate oxidation at acid pH has been shown to cleave carbohydrate vicinal hydroxyl groups without altering the structure of polypeptide chains [15]. Treatment of BGP with 0.1 mM sodium periodate, which was a rather low concentration as compared with that used in the original report [15], decreased the binding of MoAb 9-13 by more than 90% (Fig. 4). The high sensitivity to sodium periodate suggests that this epitope is carbohydrate in nature. Moreover, the proteinase K-treated BGP antigen completely lost its competitive ability (Table 2). It suggests that protein may also play a role in the reactivity of this epitope. Taking these together, we conclude that the unique human IgE-binding epitope recognized by the six lambda-type anti-BGP MoAbs is located on glycoproteins of BGP extracts. It is heat stable, and sensitive to treatments of sodium periodate and proteinase K.

Acknowledgments

This study was supported by a grant of the National Science Council, Republic of China (NSC 79-0418-B075-06).

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