

W.-D. Müller
B. Fahlbusch
K. Jung
M. Gebhardt
L. Jäger

Department of Clinical Immunology,
Friedrich Schiller University Jena, FRG

Characterization of Murine Monoclonal Antibodies to Phospholipase A₂ and Mellitin from Bee Venom

Key Words

Bee venom
Monoclonal antibodies
Phospholipase A₂
Mellitin
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Binding assay, two-site

Abstract

9 murine monoclonal antibodies (MoAbs), 7 to phospholipase A₂ (Pla₂; *Api m I*) and two to mellitin (*Api m III*) have been produced. Specificities were demonstrated using immunoblotting and enzyme immunoassays (ELISA). Antibodies specific for Pla₂ were characterized in detail. Epitope mapping of Pla₂-specific MoAbs identified three independent binding regions. 5 selected MoAbs bound to one of these sites compete with specific human IgE. Two of MoAbs characterized are suitable tools to quantify Pla₂ in a two-site binding assay.

Introduction

A relative high percentage (0.4–0.8%) of the population suffer from allergic reactions caused by insect stings (honey bee, yellow jacket) often accompanied by anaphylactic reactions [1, 2]. The knowledge about essential bee venom allergens has been improved during the last years [1, 3, 4]. Regarding IgE-mediated reactions a dominant role of a few components is obvious: hyaluronidase (*Api m II*), acidic phosphatase (*Api m IV*) and above all phospholipase A₂ (Pla₂; *Api m I*). More than 90% of all bee venom allergics produce specific IgE against Pla₂ [5, 6]. Therefore a better insight into Pla₂-specific IgE induction and regulation is of great interest and practical relevance [1]. Analysis of B (IgE, IgG, IgG4) and T cell epitopes of Pla₂ could be an essential step for a better understanding of specific IgE-triggering and modulating mechanisms. Preliminary data have been published about the structure of B cell epitopes of Pla₂. The majority of polyclonal human antibo-

dies (IgE, IgG) as well as some human monoclonal antibodies (MoAbs; IgG4) seems to recognize discontinuous epitopes in which several lysine groups appear to be critical for antibody binding [7]. Furthermore first results about T cell epitopes of Pla₂ [8–11] and mellitin [12] have been published enabling another approach to insect venom immunotherapy by peptide analogs of Pla₂ [1, 13]. The following data characterize murine MoAbs to mellitin and Pla₂ obtained after immunization of Balb/c mice with whole bee venom or pure Pla₂ and conventional fusion techniques [14, 15]. The Pla₂ specific MoAbs were analyzed regarding their binding sites, competition with human IgE and their potential application for standardization of bee venom preparations.

Correspondence to: Dr. Wolf-Dieter Müller
Department of Clinical Immunology
Friedrich Schiller University
Humboldtstrasse 3
D-O-6900 Jena (FRG)

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Material and Methods

Production of MoAbs

Female Balb/c mice (8–12 weeks old) were immunized monthly with whole bee venom (10 µg/mouse) or pure Pla₂ (Boehringer, Mannheim, FRG; 2 µg/mouse) intraperitoneally. Initially antigens were dissolved in an emulsion of aqueous venom solution and complete Freund's adjuvant (Sigma, St. Louis, Mo., USA). Cell fusion and cloning have been done as described elsewhere [14, 15]. For characterization of MoAbs supernatants [immunoblotting, direct enzyme-linked immunosorbent assays (ELISA), ELISA inhibition] as well as ammonium sulfate precipitated (50%) ascitic fluids (MoAbs labeling, epitope mapping, IgE competition, Pla₂-quantifying test) have been used. Subclass detection of MoAbs was done by a commercial kit (Boehringer, Mannheim, FRG).

Immunoblotting Experiments

Bee venom (5 mg/ml) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide gel) utilizing the Laemmli buffer system [16]. Protein bands were stained by Coomassie blue. The following molecular weight markers were used: phosphorylase b (94 kD), bovine serum albumin (BSA) (67 kD), OVA (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), α -lactalbumin c (14.4 kD). Simultaneously commercial Pla₂ (5 mg/ml, Boehringer, FRG) and mellitin (5 mg/ml, Sigma, USA) were separated for a reliable specification of MoAbs. For immunoblot studies the separated proteins were transferred electrophoretically to the NC membrane (Sartorius, FRG). Long stripes were cut from NC paper and incubated overnight with supernatants (dilution 1:20) of the different hybridomas. After washing, bound mouse antibodies were detected with biotinylated antimouse Ig (Boehringer, FRG, diluted 1:1,000 in phosphate-buffered saline (PBS), 0.5% Tween 20) and streptavidin-horseradish peroxidase (HRP) (Boehringer, FRG, diluted 1:2,000 in PBS, 0.5% Tween 20). Then the blot was washed and developed by 4-chloro-1-naphthol (Serva, FRG).

Enzyme-Linked Immunosorbent Assays

All following washing and blocking steps have been done using PBS (pH 7.2) containing 0.5% v/v Tween 20.

Direct ELISA

96-well microtiter plates (NUNC, MaxiSorp, Denmark) were coated with 50 µl of Pla₂ (5 µg/ml, Boehringer, FRG) or 50 µl of mellitin (5 µg/ml, Sigma, USA) overnight diluted in coating buffer (carbonate-bicarbonate buffer, pH 9.6). After washing and blocking a 2-hour incubation followed with 50 µl hybridoma supernatants (native, 1:10, 1:100). After washing the plates 50 µl of HRP-labeled goat antimouse globulin were added. The substrate used was *o*-phenylenediamine.

ELISA Inhibition

Briefly, microtiter plates were coated with 50 µl of Pla₂, 5 µg/ml. Before starting inhibition experiments hybridoma supernatants of Pla₂-specific MoAbs have been diluted in order to calibrate a maximum OD of 1.0–1.2. In separate tubes mixtures of a serial dilution of Pla₂ (0.1–10 µg/ml) and hybridoma supernatants were preincubated for 1 h. Then allergen-MoAb complexes were added into Pla₂-coated plates for 2 h. After washing, HRP-labeled goat antimouse globulin was added and the binding of antibodies was detected as described earlier.

Epitope Mapping of Pla₂-Specific MoAbs

First of all Pla₂-specific MoAbs were labeled with HRP and calibrated on a maximum OD of 1.0–1.2 as described above. In separate tubes mixtures of a serial dilution of each unlabeled MoAb (1:100, 1:1,000, 1:10,000) and each adjusted HRP-labeled MoAb were preincubated for 1 h. Then the probes were added into Pla₂-coated plates for 2 h. Binding of HRP-labeled MoAbs was detected as described earlier.

IgE Competition

6 sera from bee venom allergies with a high titer of specific IgE (RAST class 4) were selected. In Pla₂-coated plates a mixture of each patient serum (dilution 1:10) and a serial dilution of each MoAb (1:100, 1:1,000, 1:10,000) was incubated for 1 h. After washing biotinylated rabbit antihuman IgE (DAKO, FRG, 1:2,000) was added for 1 h. After further washing HRP-labeled streptavidin (Boehringer, 1:2,000) was added for 1 h. The test was developed as described earlier.

Detection of Pla₂ Quantities

ELISA Inhibition with MoAb 5D5. First of all MoAb 5D5 was biotinylated. Briefly 1 mg/ml of MoAb 5D5 in 0.1 M sodium bicarbonate, pH 8.4, was mixed with 50 µl *D*-biotinyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer, Mannheim; 1 mg/ml in dimethyl sulfoxide) and left for 30 min at room temperature. Unreacted biotin was removed by fast desalting column (HR 10/10, Pharmacia). The final concentration of biotinylated MoAb 5D5 was 0.5 mg/ml as judged by absorbance at 280 nm. In each case three different dilutions of biotinylated 5D5 (1:500, 1:1,000, 1:5,000) were mixed with a serial dilution of Pla₂ (10 ng/ml–10 µg/ml) and incubated for 1 h in a Pla₂-coated ELISA plate. After washing the test was continued with HRP-labeled streptavidin (1:2,000) and developed as described before.

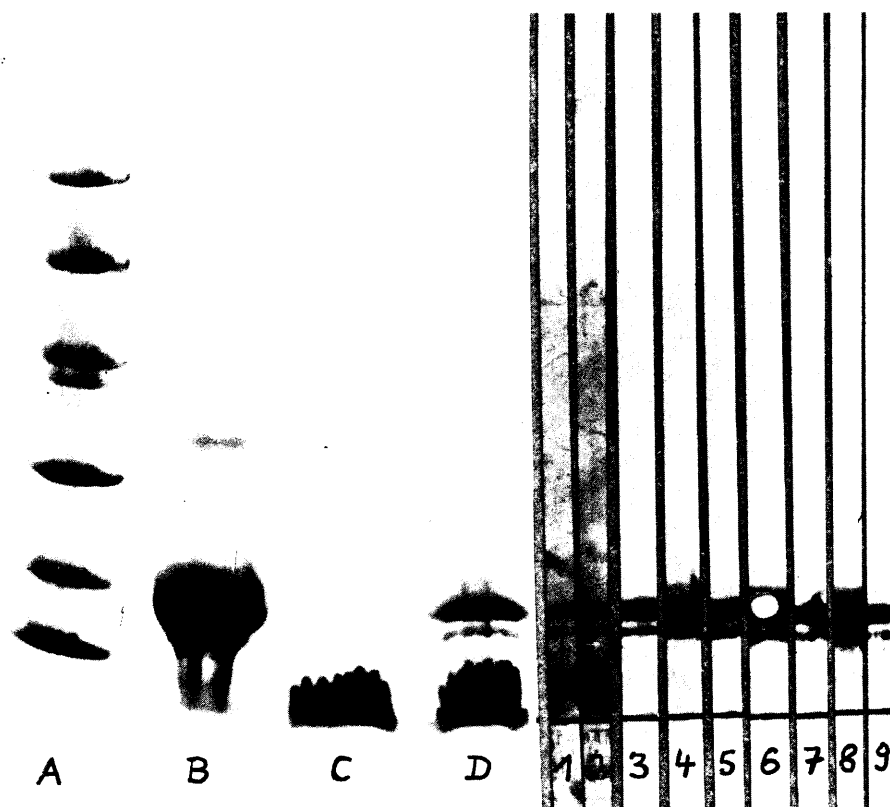
Two-Site Binding Assay. Direct detection of Pla₂ quantities from bee venom was done by a two-site solid-phase ELISA: microtiter plates were coated with 50 µl/well of capture MoAb 1C11 (20 µg/ml coating buffer) overnight at 4 °C. All following incubations were performed at room temperature for 2 h. After washing and blocking 50 µl of Pla₂ standard solution (concentration range 1–100 ng/ml) or whole bee venom (concentration range 10–1,000 ng/ml) were added to the wells. After further washing bound Pla₂ was detected using biotinylated MoAb 5D5 (1:1,000). The test was continued with HRP-streptavidin (1:2,000) and developed as described earlier.

Results

Specificities of MoAbs

9 stable hybridomas selected from two independent cell fusions were analyzed in detail. Specificities were proved by means of immunoblotting (IB) after SDS-PAGE of bee venom (fig. 1) and ELISA tests using solid phase bound and soluble Pla₂ (fig. 2–4). The IB pattern demonstrates the binding of 7 MoAbs within the typical molecular weight range of Pla₂ (fig. 1, lanes 3–9). Two other MoAbs bind to mellitin (fig. 1, lanes 1, 2). Obviously commercial batches of natural (nonsynthetic) mellitin are contaminated with traces of Pla₂ [17]. As mellitin also associates

Fig. 1. Immunoblot profiles of MoAbs to Pla_2 and mellitin after separation of bee venom by SDS-PAGE: gel pattern (A–D) of marker run (A), commercial Pla_2 (B; Boehringer, 5 mg/ml), commercial mellitin (C; SIGMA, 5 mg/ml) and bee venom (D; 5 mg/ml). IB pattern of two MoAbs to mellitin (lane 1, 2: 2F10, 3G2); IB pattern of 7 MoAbs to Pla_2 (lane 3–9: 3F3, 1D12, 3H8, 1C11, 5D5, 2B6, 2F5); used markers: phosphorylase b, 94 kD; BSA, 67 kD; OVA, 43 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 20 kD; α -lactalbumin c, 14.4 kD.



with Pla_2 very easily [17] both mellitin-specific MoAbs bound apparently to the Pla_2 impurities of mellitin (fig. 1, lane 1, 2). But undoubted specificities of MoAbs have been confirmed by ELISA tests. All MoAbs detect the solid phase-bound target antigen in a dose-dependent manner (fig. 2, 3). Only some MoAbs (5D5, 2F5) bound soluble Pla_2 (2.5 $\mu\text{g}/\text{ml}$) optimally due to some conformational alterations of the corresponding epitope (fig. 4). All 9 antibodies were tested for binding to Pla_2 as well as mellitin (solid phase and above all in solution). In this way 'artificial specificities' due to the known reciprocal impurities of commercial batches of Pla_2 and above all mellitin could evidently be ruled out. Furthermore no MoAb bound to antigen-free wells.

Epitope Mapping of Pla_2 -Specific MoAbs

Table 1 summarizes the results of epitope mapping. As expected a distinct inhibition was found if the homologous MoAb was used. Binding of MoAbs 5D5 and 3F3 was not significantly inhibited by all other antibodies. On the other hand, the remaining 5 MoAbs seem to detect the same or neighboring sites. Summing up the epitope mapping the panel of Pla_2 -specific MoAbs seems to recognize at least three different binding sites on solid phase-bound Pla_2 :

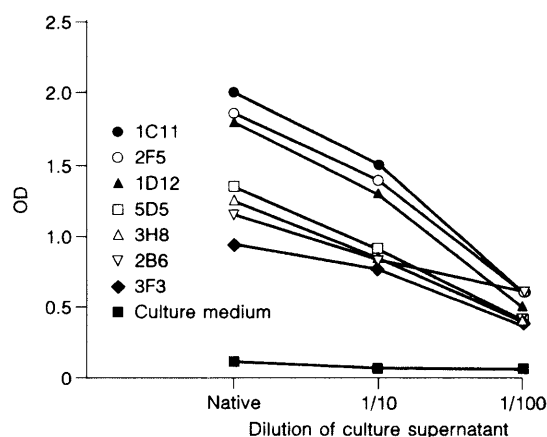


Fig. 2. Direct ELISA (solid phase: 5 $\mu\text{g}/\text{ml}$ Pla_2) of 7 MoAbs using different dilutions of hybridoma supernatants [none of the tested MoAbs bound to mellitin-coated or antigen-free wells; data not shown].

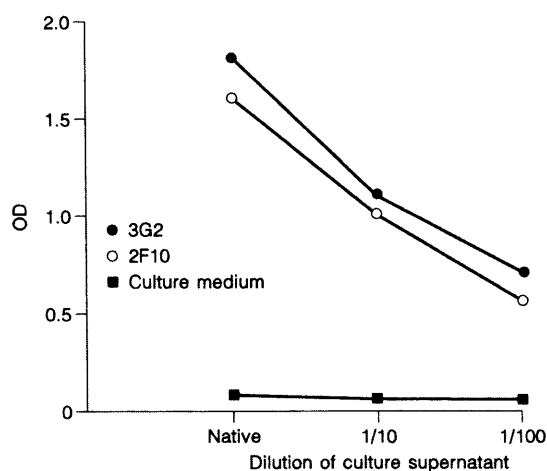


Fig. 3. Direct ELISA (solid phase: 5 µg/ml mellitin) of 2 MoAbs using different dilutions of hybridoma supernatants [none of the tested MoAbs bound to Pla₂-coated or antigen-free wells; data not shown].

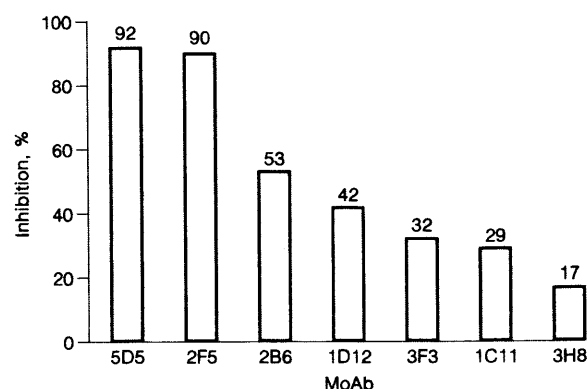


Fig. 4. Maximum inhibition of binding of 7 MoAbs to solid phase-bound Pla₂ (5 µg/ml) by soluble Pla₂ (in each case 2.5 µg/ml) in an ELISA inhibition test [no inhibition by equivalent doses of mellitin was found; data not shown].

Table 1. Epitope mapping of MoAbs specific for Pla₂

	5D5 ^a	3F3 ^a	2F5 ^a	2B6 ^a	1D12 ^a	3H8 ^a	1C11 ^a	Site
5D5 (IgG1)	+++	(+)	(+)	(+)	(+)	(+)	(+)	A
3F3 (IgG3)	(+)	+++	(+)	(+)	(+)	(+)	(+)	B
2F5 (IgG1)	(+)	(+)	+++	++	++	++	++	C/C'
2B6 (IgG1)	(+)	(+)	+++	+++	+++	+++	+++	C/C'
1D12 (IgG1)	+	(+)	+++	+++	+++	+++	+++	C/C'
3H8 (IgG1)	+	(+)	+++	+++	+++	+++	+++	C/C'
1C11 (IgG1)	(+)	(+)	++	+++	+++	+++	+++	C/C'

Binding sites A, B, C are defined by their association with MoAbs which compete with each other. (+) = <25% inhibition; + = 25–49% inhibition; ++ = 50–74% inhibition; +++ = >75% inhibition.

^a Biotinylated MoAbs.

site A (5D5), site B (3F3) and the 'splitted' site C/C' (2B6, 2F5, 3H8, 1D12 and 1C11). Consequently in this experiment sites A, B, C/C' are defined by their association to various MoAbs which compete with each other.

IgE Competition

Table 2 shows the data of IgE inhibition studies. 6 sera (in each case dilution 1:10) from bee venom allergics were selected according to their high titer of bee venom-specific IgE (RAST class 4). The inhibitory capacity of Pla₂-specific MoAbs varied. Whereas MoAbs 5D5 (= site A) and 3F3 (= site B) were almost ineffective, the other 5 MoAbs identifying site C/C' compete with human IgE binding to

Pla₂. Obviously site C/C' represents structures of Pla₂ participating in human IgE binding.

Selection of MoAbs to Quantify Pla₂

Binding of our MoAbs to at least 3 different sites of Pla₂ is a favorable prerequisite to quantify the bee venom marker allergen especially for standardization purposes. First of all MoAb 5D5 was selected because of its excellent binding to soluble Pla₂ (see fig. 4). Therefore directly labeled (biotinylated) MoAb 5D5 was tested in an ELISA inhibition, using biotinylated MoAb, solid phase-bound Pla₂ and soluble Pla₂ in different concentrations. In this test the optimal dilution of 5D5 biotinylated was 1:10³ with

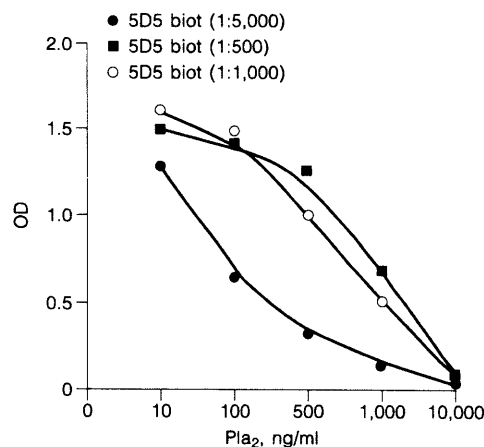


Fig. 5. Inhibition of binding of biotinylated (biot) MoAbs 5D5 (various dilutions) to solid phase-bound Pla₂ (5 µg/ml) by soluble Pla₂ (10 ng/ml to 10 µg/ml).

the strongest slope of the inhibition curve (fig. 5). After a series of tests with different capture antibodies it was obvious that the combination 1C11/5D5 biotinylated gives the best results. Figure 6 demonstrates titration curves for pure Pla₂ as standard compared with bee venom. Both curves are almost the same if whole bee venom was used in a concentration 10 times higher than pure Pla₂. This agrees very well with the medium Pla₂ content (12%) in bee venom [3].

Discussion

Contrary to other widespread allergens (e.g. from grass pollen) the allergenic components of bee venom are well known and the most important are characterized according their amino acid sequences [1]. Recent investigations focus on IgE inducing and regulating mechanisms of B and T cells at the epitope level [7, 8, 10, 11, 13]. We produced Pla₂-specific MoAbs for identification and standardization of the allergen on one hand and investigations of epitope specificities on the other. After immunization with whole bee venom only three useful MoAbs, one to Pla₂ (5D5) and two to mellitin (2F10, 3G2) were obtained. Further 6 Pla₂ binding MoAbs were added after immunization with pure Pla₂. Specificities of MoAbs were identified by IB after SDS-PAGE (fig. 1). Data on antibodies specific for Pla₂ and mellitin bound within the expected molecular ranges were also published by other authors [3]. In the case of Pla₂ MoAbs the double labeling is due to an-

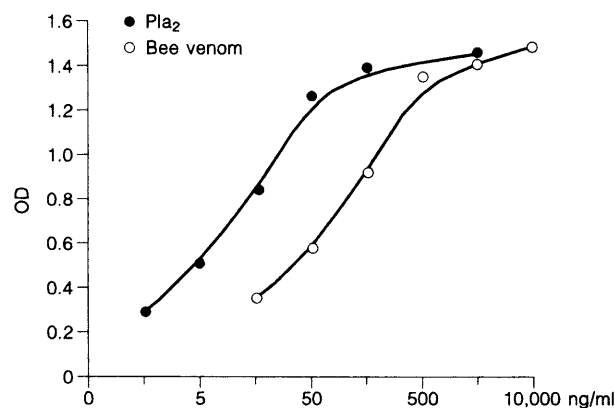


Fig. 6. Two-site binding assay for pure Pla₂ compared to whole bee venom (capture MoAb 1C11, indicator biotinylated MoAb 5D5, dilution 1:1,000).

Table 2. Maximum inhibition (in %) of binding of specific human IgE by MoAbs specific for Pla₂ in an ELISA competition test (5 µg/ml Pla₂ on solid phase; all 6 patient sera tested were diluted 1:10)

Patient No.	2A10	Site A	Site B	Site C/C'				
		5D5	3F3	2B6	2F5	3H8	1D12	1C11
1	10	5	0	52	37	29	43	33
2	7	8	2	49	38	27	39	30
3	12	7	0	37	24	26	28	40
4	3	0	3	47	42	37	40	47
5	12	9	0	39	29	19	29	29
6	3	14	0	24	20	0	0	0

2A10 = Irrelevant control MoAb (IgG1), specific for apolipoprotein A₁.

tibody binding to both 'isoforms' of Pla₂, a glycosylated (88%) and an unglycosylated one (12%). This result refers to the binding to protein structures only. The diffuse binding pattern of mellitin antibodies within a molecular range of about 3–12 kD is the consequence of simultaneous presence of mono-, di- and tetrameric mellitin in bee venom [1, 2]. Apparent labeling of protein bands in the molecular range of Pla₂ by mellitin MoAbs based on the property of mellitin combining to Pla₂ impurities always contained in commercial batches of mellitin [17].

Because of the minor allergological relevance of mellitin only a few orienting tests have been done with MoAbs

2F10 and 3G2. Epitope mapping shows that both MoAbs recognize the same epitope which also binds human IgE [data not shown]. This result is not surprising considering the low number of potential B cell epitopes of mellitin [18].

All other investigations deal with Pla₂ antibodies. ELISA tests confirm the specificities. The variable binding to solid phase-bound and soluble Pla₂ could be due to the different accessibility of the corresponding epitopes. Similar differences have been found with certain MoAbs we produced against grass pollen allergens [15, 19].

Epitope mapping identified at least three independent binding regions (A, B, C/C'). 5 of 7 MoAbs detect identical or at least neighboring epitopes (site C/C'). Interestingly this site seems to participate in the binding of specific human IgE. Because of the availability of MoAbs specific for different epitopes of Pla₂ an antibody combination to quantify Pla₂ in a two-site binding assay was developed. As indicator antibody MoAb 5D5 was used due to its excel-

lent binding to soluble Pla₂. A detailed testing of all potential captured MoAbs identified 1C11 as the best one. The two-site binding assay shows an optimal range from 5 to 50 ng/ml Pla₂ and a detectable concentration of 2–3 ng/ml Pla₂. The nearly parallel slope of the bee venom curve if bee venom is used in equivalent (10 times higher) concentrations confirms the usefulness of the assay and mirrors the Pla₂ content in bee venom very well. Now detailed investigations of this two-site binding assay tend to optimize the test and to compare different sources of bee venom preparations regarding their content of marker allergen Pla₂. Interestingly a recent publication [20] reports on a notable variability of Pla₂ concentrations in bee venoms. Furthermore our immunological test represents a good alternative to complicated and time-consuming biochemical or functional bioassays for Pla₂ activities [3, 21] in bee venom.

References

- King TP: Insect venom allergens; in Baldo BA (ed): *Molecular Approaches to the Study of Allergens*. Monogr Allergy. Basel, Karger, 1990, vol 28, pp 84–100.
- Kroegel C: Insektenstiche. Pathomechanismus und Therapie. *Med Monatsschr Pharm* 1990;6: 172–182.
- Einarsson R: Components in insect venoms and analytical methods. *Allergologie* 1986 (suppl 1):24–29.
- Diener C, Jäger L: Analysis and standardization of bee venom in vitro. *Allergologie* 1986 (suppl 1):30–33.
- Kemeny DM, Harris MG, Youten LJF, MacKenzie-Mills M, Lessof MH: Antibodies to purified bee venom proteins and peptides. I. Development of a high specific RAST for bee venom antigens and its application to bee sting allergy. *J Allergy Clin Immunol* 1983;71:505–514.
- Kemeny DM, Lessof MH: Immunotherapy in insect venom allergy; in Lessof MH, Lee TH, Kemeny DM (eds): *Allergy: An International Textbook*. Chichester, Wiley, 1987, pp 631–639.
- Schneider T, Dudler T, Lang AB, Sippl MI, Cramer R, Suter M: Epitope mapping on bee venom phospholipase A₂ molecule recognized by human IgE or IgG4 antibodies; in Kraft D, Sehon AH (eds): *Molecular Biology and Immunology of Allergens*. Boca Raton, CRC Press, in press.
- Carini C, Iwata M, Warner J, Ishizaka K: A method to generate antigen-specific suppressor T cells in vitro from peripheral blood T cells of honey bee venom-sensitive, allergic patients. *J Immunol Methods* 1990;127:221–233.
- Lesourd B, Paupe J, Thiollot M, Moulis R, Sainte-Landy J, Scheinmann P: Hymenoptera venom immunotherapy. I. Induction of T cell-mediated immunity by honey bee venom immunotherapy: Relationships with specific antibody responses. *J Allergy Clin Immunol* 1989; 83:563–570.
- Dhillon M, Roberts C, Nunn T, Kuo M: Mapping human T cell epitopes on phospholipase A₂: The major bee venom allergen. *J Allergy Clin Immunol* 1992;90:42–51.
- Carballido JM, Carballido-Perrig N, Terres G, Heusser CH, Blaser K: Bee venom phospholipase A₂-specific T cell clones from human allergic and nonallergic individuals: Cytokine patterns change in response to the antigen concentration. *Eur J Immunol* 1992;22:1357–1363.
- Fehlner PF, Berg RH, Tam JP, King TP: Murine T cell response to mellitin and its analogs. *J Immunol* 1991;146:799–806.
- Litwin A, Pesce AJ, Michael JG: Regulation of the immune response to allergens by immunosuppressive allergenic fragments. I. Peptic fragments of honey bee venom phospholipase A₂. *Int Arch Allergy Appl Immunol* 1988;87:361–366.
- Müller WD, Diener C, Jung K, Jäger L: Antigens of timothy and other grass pollen extracts identified by monoclonal antibodies. *Allergol Immunopathol* 1988;16:315–320.
- Fahlbusch B, Müller WD, Diener C, Jäger L: Detection of cross-reactive determinants in grass pollen extracts using monoclonal antibodies against group IV and group V allergens. *Clin Exp Allergy* 1993;23:51–60.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- Fletcher JE, Michaux K, Jiang MS: Contribution of bee venom phospholipase A₂ contamination in mellitin fractions to presumed activation of tissue phospholipase A₂. *Toxicon* 1990;28:647–656.
- King TP, Kochoomian L, Joslyn A: Mellitin-specific monoclonal and polyclonal IgE and IgG1 antibodies from mice. *J Immunol* 1984; 133:2668–2673.
- Müller WD, Fahlbusch B, Schlenvoigt G, Wahl R, Jäger L: Qualitative and quantitative detection of group V and IV grass pollen allergens using specific monoclonal antibodies; in Kraft D, Sehon AH (eds): *Molecular Biology and Immunology of Allergens*. Boca Raton, CRC Press, in press.
- Schumacher MJ, Schmidt JO, Egen NB, Dillon KA: Biochemical variability of venoms from individual European and Africanized honey bees (*Apis mellifera*). *J Allergy Clin Immunol* 1992;90/1:59–65.
- Farooqui AA, Horrocks LA: Determination of phospholipases, lipases, and lysophospholipases; in Boulton GB, Harrocks LA (eds): *Lipids and Related Compounds*. Clifton, The Humana Press, 1988, pp 179–209.