

Comparison of the Antibody Response to Bee Venom Phospholipase A₂ induced by Natural Exposure in Humans or by Immunization in Mice

Theres Schneider,^{1*} Thomas Dudler,^{1*} Robert R. Annand,² Michael H. Gelb,² Te P. King³ and Mark Suter^{1†}

¹ Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

² Departments of Chemistry and Biochemistry, University of Washington, Seattle, WA, USA

³ The Rockefeller University, New York, NY, USA

Two human and twelve murine monoclonal antibodies directed against the main bee venom allergen phospholipase A₂ (PLA) were evaluated for their fine specificity of binding to antigen and their ability to inhibit the enzymatic activity of the antigen. Antibodies were induced by natural exposure of beekeepers to bee venom or immunization of mice via different methods. Both human monoclonal antibodies (hmAbs) were previously shown to recognize the native three-dimensional conformation of PLA and are directed against discontinuous epitopes which include lysine residue at position 25 as a contact residue. In contrast, six of the murine monoclonal antibodies (mmAbs) bind to the denatured structure of the protein as determined by enzyme-linked immunosorbent assay. The epitopes recognized are located near the C-terminal end (n=8), in the centre of the polypeptide (n=1), near the N-terminal end (n=1) or include the carbohydrate part (n=2) of the PLA molecule. The capacity of the antibodies to modify the enzymatic activity was also determined. The hmAbs significantly inhibit the enzyme (70–79%), whereas the mmAbs produced various degrees of inhibition (39–100%). Since the X-ray structure of PLA is known, the epitopes can be visualized in the context of the three-dimensional structure of the antigen. A qualitative correlation was found between the location of epitopes and the inhibition pattern. Strong inhibition was seen with those antibodies that recognize epitopes that lie on the surface of the enzyme that is thought to contact the phospholipid bilayer. The results show that even though both hmAbs and most mmAbs inhibit the enzymatic activity of PLA, the antigen-binding properties of antibodies from different species raised after different routes of immunization differ significantly. Thus, detailed epitope mapping studies using murine antibodies prepared by artificial immunization may have limited value in predicting epitope patterns relevant to an antibody response to allergens in humans naturally exposed to antigen/allergen. © 1997 John Wiley & Sons, Ltd.

J. Mol. Recogn. **10**, 93–100 (1997) No. of Figures: 3 No. of Tables: 3 No. of References: 38.

Keywords: monoclonal antibodies; human; antigen; mouse; allergen; phospholipase; epitope mapping

Received 17 January 1996; revised 10 June 1996; accepted 26 June 1996

Introduction

Following an encounter of an individual with a foreign antigen, antibodies against the antigen may exert diverse biological responses including the neutralization of bio-

logically active antigens or infectious particles, complement fixation, or binding of antigen-antibody complexes to Fc receptors on cell surfaces to augment defence mechanisms of the immune system. In addition, antibodies may exert adverse biological effects exemplified in allergic diseases. Distinct regions of antigens that contact antibodies are called epitopes (Getzoff *et al.*, 1987). It is assumed that the identification of these epitopes and a thorough understanding of how epitopes on the antigen interact with complementary regions on antibodies (paratopes) (Hopp, 1986; Berzofsky, 1985; Geysen *et al.*, 1987b; Alexander *et al.*, 1992) may serve as a basis for the development of vaccines that induce antibodies to specific epitopes on antigens (Bahraoui *et al.*, 1990; Martin *et al.*, 1991; Ho *et al.*, 1991). Furthermore, precise knowledge of antigen-antibody interactions may be useful in defining algorithms that predict epitopes from the primary structure of protein antigens or in defining antigenicity of specific molecules.

The strategies for mapping antibody epitopes differ considerably. They include: (1) assays of the binding of antibody to a set of overlapping short peptides that together span the entire antigen (Geysen *et al.*, 1987a); (2)

* Current address: Departments of Chemistry and Biochemistry, BG-10, University of Washington, Seattle, WA 98195, USA.

† Correspondence to: M. Suter, Institute of Virology, Winterthurestrasse 266a, CH-8057, Zürich, Switzerland.

Contract grant sponsor: Swiss National Science Foundation, contract grant number: NSF 31-28815.90; 31-40661.34; BBW 94.0154

Contract grant sponsor: National Institutes of Health; contract grant number: HL-36235

Abbreviations used CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; hmAb, human monoclonal antibody; i.p., intraperitoneal; mmAb, murine monoclonal antibody; OD, optical density; PBS, phosphate-buffered saline, 10 mM; PLA, phospholipase A₂ from honey bee venom (*apis mellifera*); rPLA, recombinant PLA; rPLA dd 1–19, recombinant PLA molecule missing amino acids 1–19; rPLA dd 73–78, recombinant PLA molecule missing amino acids 73–78; rPLA dd 99–118, recombinant PLA molecule missing amino acids 99–118; rPLA K25E, recombinant PLA variant with lysine 25 exchanged by glutamate; rPLA K85E, recombinant PLA variant with lysine 85 exchanged by glutamate.

comparison of antibody binding to cognate antigen *versus* antigen fragments of closely related proteins (Cunningham *et al.*, 1989); (3) determination of amino acid residues of the antigen that are critical for binding to antibody by employing site-directed mutagenesis (Jin *et al.*, 1992; Dudler *et al.*, 1994) or amino acid degradation (Yuan and Low, 1992); and (4) visualization of antigen-antibody complexes by X-ray crystallography, cryoelectron microscopy or nuclear magnetic resonance spectroscopy (Stanfield *et al.*, 1990; Smith *et al.*, 1993; Zinn-Justin *et al.*, 1993). The antibodies used for such studies are often raised in rodents by artificial immunization with the protein of interest. The results thus obtained are generalized to include other species and are considered to be a particular feature of the specific antigen (Martin *et al.*, 1991; Strasburger *et al.*, 1989; Zhang *et al.*, 1992; Ohara *et al.*, 1993; Stüber *et al.*, 1990a). But there might be differences in the epitopes recognized by antibodies from different species on the one hand (Söderlind *et al.*, 1993) and between antibodies raised upon natural exposure to foreign antigen or induced by immunization with adjuvant on the other hand (Boyer *et al.*, 1991).

To address these issues directly, we have compared the epitopes on phospholipase A₂ (PLA), the main allergen present in bee venom (Kuchler *et al.*, 1989; Scott *et al.*, 1990a), recognized by human monoclonal antibodies (hmAbs) (Schneider *et al.*, 1994b) or murine monoclonal antibodies (mmAbs) (Müller *et al.*, 1993). Two hmAbs of the IgG₄ isotype were made from lymphocytes of a non-allergic beekeeper naturally exposed to bee venom. These hmAbs were shown to be part of the polyclonal immune response of beekeepers who have been exposed for years to bee venom via bee stings (Dudler *et al.*, 1994; Schneider *et al.*, 1994a, 1994b). γ -Globulins from such individuals were previously shown to be protective when given to bee venom allergic individuals before exposing them to bee venom (Lessof *et al.*, 1978; Müller *et al.*, 1986; Bousquet *et al.*, 1987). Therefore, hmAbs against PLA representative of the polyclonal and possibly protective immune response against this allergen have been studied in some detail. Binding of both hmAbs to PLA was shown to depend on the correct three-dimensional structure of the antigen. Additionally, the asparagine-linked carbohydrate chain found on naturally occurring PLA is not part of the epitope for these hmAbs (Schneider *et al.*, 1994a, 1994b). The hmAbs failed to bind to the enzymatically active point mutant of PLA in which lysine at position 25 was changed to glutamate or glutamine. This lysine residue is thus considered to be part of the epitope for both human monoclonal and part of the polyclonal immune response in beekeepers directed against PLA (Dudler *et al.*, 1994). Besides antigen binding, another biological function was assayed, the ability of the hmAbs to inhibit the enzymatic activity of PLA.

In this report, the two hmAbs described above were compared to several mmAbs obtained after immunization of mice (Müller *et al.*, 1993) with PLA. A panel of PLA variants were prepared to map the epitopes recognized by the mmAbs and hmAbs. In addition, the effect of these antibodies on the enzymatic activity of PLA was analysed. Since the high resolution X-ray structure of PLA is available (Scott *et al.*, 1990a), the epitopes can be visualized in the context of the three-dimensional structure of the

antigen. The data indicate that hmAbs and mmAbs recognize different epitopes present on PLA, and these antibodies modulate the enzymatic activity to various extents depending on the epitope.

Experimental

PLA-specific antibodies

HmAbs BVA1 and BVA2 specific for PLA were secreted by two stable and independent hybridoma lines which were established as described (Schneider *et al.*, 1994b). Briefly, peripheral blood mononuclear cells of a beekeeper was transformed with Epstein Barr virus. PLA-specific lymphoblastoid cells were further fused with human heteromyeloma F3B6 or LA55. The hybridomas were tested for secretion of anti-PLA antibodies and cloned by limiting dilution.

MmAbs 5D5, 3F3, 2F5, 1D12, 3H8, IC11 and 2B6 were produced in female Balb/c mice immunized i.p. with PLA emulsified with complete Freund's adjuvant (CFA) as described (Müller *et al.*, 1993).

MmAb 1B76: a Balb/c mouse was immunized i.p. at weeks 0, 3 and 9 with 200 μ l of 20 μ g/ml PLA and 5 mg/ml alum in 0.05 M sodium phosphate, pH 6.4

MmAbs 830716 and 8307175: an AKR mouse was immunized i.p. at weeks 0, 3, 5 and 8 with 200 μ l of 20 μ g/ml PLA in 0.05 M sodium phosphate emulsified 1:1 with CFA and subsequently at weeks 13, and 14 with 200 μ l of 20 μ g/ml PLA and 5 mg/ml alum in 0.05 M sodium phosphate, pH 6.4.

MmAbs 831108 and 831115: a Balb/c mouse was immunized i.p. at weeks 0, 3 and 5 with 200 μ l of 20 μ g/ml PLA and 5 mg/ml alum in 0.05 M sodium phosphate, pH 6.4.

For 1B76, 830716, 8307175, 831108 and 831115 the fusion with sp 2/0 cells was made 3 days after the last immunization.

PLA preparations

Natural PLA was purchased from Boehringer Mannheim.

Deglycosylated PLA was prepared as follows: PLA (Boehringer Mannheim, 500 μ g) was dissolved in 1 ml of 250 mM sodium acetate, pH 6.5 containing 1 mM phenylmethylsulfonyl fluoride, 1 unit endoglycosidase F/N-glycosidase F (Boehringer Mannheim) was added, and the mixture was incubated for 24 h at 37°C. Deglycosylated PLA was dialysed against PBS, pH 7.4 and stored at -20°C until use.

PLA acetylated at lysine residues was prepared as described previously (Schneider *et al.*, 1994a). Briefly, 1 mg PLA (Boehringer Mannheim) was dissolved in 1 ml of 100 mM NaHCO₃, pH 8.5, and 1 mg of sulphonate-NHS-acetate (Pierce, Rockford, IL, USA), dissolved in 100 μ l DMSO, was added. The reaction was kept for 1 h at room temperature, and the sample was extensively dialysed against PBS, pH 7.4. This procedure was shown to result in

an exhaustive acetylation of the ϵ -amino group of lysines (Schneider *et al.*, 1994a).

Recombinant PLA (rPLA) was expressed in *E. coli* as fusion protein with six histidine residues linked to the N-terminus (Dudler *et al.*, 1992). The production of S-sulphonated rPLA and the variants rPLA K25E and K85E is described elsewhere (Dudler *et al.*, 1992 and 1994). The truncated antigen variant rPLA dd 1–19 was constructed by subcloning a gene fragment encoding PLA residues 20–134 into *Bam*HI and *Hind*III restriction sites of the pDS56/RBSII 6 $\times$ His expression plasmid (Dudler *et al.*, 1992; Stüber *et al.*, 1990b). This gene fragment was generated by PCR using the primers 5'-CCGGATCCGAACTCGGCC-3' and T7 (Stratagene) and the synthetic gene encoding the full-length PLA polypeptide (Dudler *et al.*, 1992) in a pBluescript II SK vector (Stratagene) as a target. PCR was performed in 100 μ l of sample containing the appropriate buffer, 200 μ M of each deoxy nucleotide triphosphate, 0.5 μ M of both primers, 10 ng of target DNA and 1 U Vent DNA polymerase (New England Biolabs, Schwalbach/Taunus, Germany). The samples were subjected to two cycles of amplification consisting of denaturation for 1 min at 95°C, annealing for 1 min at 40°C and extension for 1 min at 72°C, followed by 25 cycles under identical conditions except that the annealing was done at 50°C. The PCR product was purified by agarose gel electrophoresis and subcloned into *Bam*HI and *Hind*III restriction sites of the expression plasmid. The truncated variants rPLA dd 73–78 and dd 99–108 were generated by excision of internal gene segments from the full-length PLA expression construct (Dudler *et al.*, 1992). For rPLA dd 73–78, the expression plasmid was digested with *Aff*II and *Eco*RV, and cohesive ends were filled with Klenow DNA polymerase. Gel-purified plasmid was recircularized with ligase, resulting in an in-frame fusion between amino acid residues 72 and 79. For deletion variant rPLA dd 99–118, the *Xho*I/*Sal*I fragment was excised from the expression plasmid followed by religation of the compatible cohesive ends of the purified plasmid, leading to an in-frame fusion between amino acid residues 98 and 119. The correct sequence of all deletion constructs was confirmed by nucleotide sequencing, and truncated PLA variants were produced as described for full length PLA (Dudler *et al.*, 1992, 1994).

Determination of the enzymatic activity of natural and modified PLA

Enzymatic activity of natural and modified PLA was determined using a spectrophotometric assay with the short-chain phospholipid substrate racemic 1,2-bis(hexanoylthio)propyl-3-phosphomethanol (Yuan *et al.*, 1990).

The effect of antibody binding on enzyme activity was determined in a pH stat scooting assay with 230 μ M 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol lithium salt vesicles in the presence of 1 μ M polymyxin B, 600 μ M CaCl₂, 1 mM NaCl, pH 8.0 at 21°C (Dudler *et al.*, 1994; Jain and Gelb, 1991). PLA (100 ng) was mixed with various molar ratios of monoclonal antibodies in PBS, pH 7.4 in a total volume of 10 μ l and incubated for 1 h at room temperature. A portion of this mixture (7.5 μ l) was added to the assay solution, and the initial rate of substrate hydrolysis

was determined by monitoring the consumption of 3 mM NaOH.

Enzyme-linked immunosorbent assay (ELISA)

Binding ELISA and inhibition ELISA were performed as described (Schneider *et al.*, 1994b). Briefly, polystyrene microtitre plates (Maxisorp, NUNC, Roskilde, DK) were coated with 5 μ g/ml of antigen in PBS, pH 8.0. Remaining free sites were then blocked with blocking buffer consisting of 1% casein hydrolysate (Oxoid Ltd, Basingstoke, Hampshire, UK) or 2% low-fat dry milk, respectively, and 5% Tween20 in PBS, pH 7.4.

For the binding ELISA, antibodies diluted in blocking buffer were then added to the antigen-coated plates, and the antibodies bound were determined using an appropriate detection system.

For the inhibition ELISA, antibodies appropriately diluted in blocking buffer were preincubated with different amounts of *fluid-phase antigen*. Thereafter the antigen-antibody mixture was transferred to the antigen-coated and blocked plates. The antibodies bound to the solid phase were then analysed by the appropriate detection system.

Results

HmAbs and mmAbs are specific for PLA

The specificity of the hmAbs and mmAbs for PLA was evaluated by binding and inhibition ELISA. The two hmAbs as well as all 12 mmAbs bound to PLA-coated microtitre plates (Fig. 1A and 1B and Table 1). This was expected because binding to PLA attached to solid phase was used as a positive selection criterion in the screening of the monoclonal antibodies (Schneider *et al.*, 1994b; Müller *et al.*, 1993). To analyse whether the 14 antibodies are directed against the native conformation of PLA, they were assayed by inhibition ELISA. Both hmAbs recognized PLA present in the fluid phase. In contrast, the binding of only four of the 12 mmAbs to solid-phase PLA was inhibited by fluid-phase PLA by more than 50%. Representative data from inhibition ELISA analysis are shown in Fig. 1C and 1D. Numerical data of all monoclonal antibodies are given in Table 1.

Most mmAbs recognize non-glycosylated PLA

To analyse whether the asparagine-linked sugar chain of PLA is part of the epitopes recognized by the mmAbs, the binding of these antibodies to PLA and its deglycosylated variant was compared by binding ELISA. As shown in Table 1, both hmAbs and 10 of the mmAbs bound equally to glycosylated and non-glycosylated antigen, whereas two mmAbs bound only very weakly to deglycosylated PLA.

Half of the mmAbs recognize denatured PLA

To evaluate whether some mmAbs are able to recognize unfolded protein antigen, mmAbs were assayed using natural PLA or S-sulphonated rPLA in a binding ELISA. S-

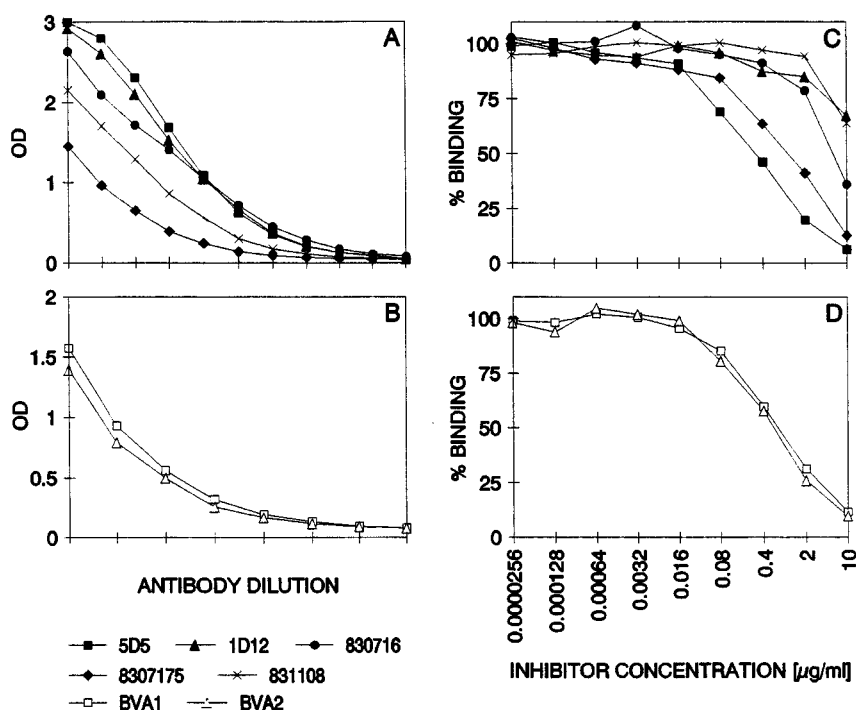


Figure 1. PLA specificity of mmAbs and hmAbs assayed with solid-phase bound or fluid-phase PLA. A: binding ELISA with five representative mmAbs serially twofold diluted; the starting point dilutions were 290 ng/ml (5D5), 230 ng/ml (1D12), 228 ng/ml (830716), 220 ng/ml (8307175), 240 ng/ml (831108). B: binding ELISA with BVA1 and BVA2. The supernatants were serially twofold diluted starting at 1:800 (BVA1) or 1:500 (BVA2), respectively. C: inhibition ELISA with five representative mmAbs. The mmAbs were used at a suboptimal dilution. D: inhibition ELISA with BVA1 and BVA2. The supernatants were used at a dilution of 1:1600 (BVA1) or 1:1000 (BVA2), respectively. In A and B the antibody dilutions are given along the abscissa and the resulting OD 405 nm values measured after addition of secondary antibody are given along the ordinate. In C and D the inhibitor concentration is plotted against the remaining percentage of binding to solid-phase bound PLA.

Table 1. Binding of hmAbs and mmAbs to solid and fluid phase PLA preparations

Group*	Antibody	PLA native	Solid phase [†]		Fluid phase [‡]	
			PLA deglycosylated	rPLA S-sulphonated	PLA native	
I	BVA1	100	100	4	88	
	BVA2	100	100	4	90	
	830716	100	100	3	62	
II	5D5	100	100	5	94	
III	3F3	100	100	91	6	
	1D12	100	100	100	37	
	3H8	100	100	100	11	
	1C11	100	100	100	44	
	2B6	100	100	100	33	
IV	8307175	100	100	100	88	
	2F5	100	100	13	56	
	1B76	100	100	3	25	
V	831108	100	8	2	42	
	831115	100	12	2	40	

* All monoclonal antibodies were grouped on the basis of their differential antigen binding patterns given in Table 2.

[†] The binding of hmAbs and mmAbs to solid-phase-bound deglycosylated or sulphonated PLA is given as a percentage of the binding measured with solid-phase native PLA.

[‡] The inhibition of the binding to solid-phase bound native PLA with 10 $\mu\text{g/ml}$ fluid-phase native PLA assayed in an inhibition ELISA at a suboptimal antibody concentration is given as a percentage of the binding measured with solid-phase native PLA.

sulphonated rPLA displays a denatured structure, and it lacks the cross-linking disulphide bonds of the native PLA (Dudler *et al.*, 1992).

No binding to S-sulphonated rPLA was observed with the hmAbs. Similarly, six mmAbs bind weakly or not at all to S-sulphonated rPLA, and six mmAbs show equivalent binding to PLA and S-sulphonated rPLA (Table 1). These former mmAbs probably recognize discontinuous, conformation-dependent epitopes, whereas the latter probably recognize continuous epitopes on PLA.

Production and analysis of rPLA fragments

To further define epitopes recognized by the mmAb a panel of rPLA variants was produced (Fig. 2). All of the rPLA variants (Fig. 2) were expressed in *E. coli* purified and refolded as described (Dudler *et al.*, 1992). The protein preparations were >95% pure as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and, as expected, truncated PLA variants displayed an increased electrophoretic mobility compared to the full-length molecule (data not shown). In a previous report we have shown that rPLA variants with single amino acid substitutions display a native-like three-dimensional structure as determined by various biochemical and enzymatic tests (Dudler *et al.*, 1994; Yuan *et al.*, 1990). All truncated rPLA molecules were soluble in physiological buffers. rPLA dd 99–118 displays full enzymatic activity on the short-chain phospholipid substrate and thus has a native-like conformation. This result is consistent with the X-ray structure of PLA which shows that the segment amino acids 99–118 form an external loop that is not part of the catalytic site. In contrast, rPLA dd 1–19 and rPLA dd 73–78 display no measurable enzymatic activity probably because these segments are required for the proper folding of PLA.

Fine specificity of the epitopes recognized by mmAbs

rPLA dd 1–19, dd 73–78, dd 99–118, K25E, K85E and acylated PLA produced by chemical modification of natural PLA were used to evaluate further the binding of mmAbs or hmAbs using a binding ELISA (Fig. 3 and Table 2). It was found that the monoclonal antibodies could be divided into

five groups based on differential antigen-binding patterns. Group I includes both hmAbs (BVA1 and BVA2) and one mmAb (830716). All three fail to bind to acylated PLA, and mutation of PLA lysine 25 but not lysine 85 to glutamate reduces antibody binding. Moreover, deletion of the C-terminal loop of PLA (rPLA dd 99–118) does not effect monoclonal antibody binding, but deletion of amino acids 1–19 and 73–78 does. This is further evidence that group I antibodies recognized only a native-like structure. Group II is composed only of mmAb 5D5. The binding of this mmAb is only modestly affected by antigen acylation, mutation of lysine 85 does not decrease binding and mutation of lysine 25 effects binding only modestly. In addition, 5D5, unlike group I antibodies, fails to recognize rPLA dd 99–118, but it does recognize rPLA dd 1–19. Group III includes some of the mmAbs that recognize denatured PLA (3F3, 1D12, 3H8, 1C11 and 2B6). These antibodies fail to bind acylated PLA and rPLA dd 99–118. The binding of group III antibodies to PLA is not effected by either lysine point mutation or deletion of residues 1–19 or 73–78, but deletion of the amino acids 99–118 impairs antibody binding. Group IV antibodies (8307175, 2F5 and 1B76) show the same binding features as group III mmAbs except they still bind to acylated PLA. The fact that group III and IV antibodies bind to rPLA dd 1–19 and rPLA dd 73–78 is further evidence that they can recognize unfolded PLA. Finally, group V includes mmAbs 831108 and 831115. These fail to bind to all PLA molecules that lack the carbohydrate moiety (Table 2 and data not shown).

HmAbs and mmAbs inhibit the enzymatic function of PLA

The monoclonal antibodies were probed for their capacity to inhibit the enzymatic activity of PLA (Table 3). Native PLA was preincubated with various amounts of antibodies and the mixture was then added to a vesicle assay of PLA. All antibodies caused inhibition of the enzymatic activity. In general, the antibodies which recognize the correct three-dimensional structure or the sugar residues are the most potent inhibitors. Three mmAbs (3F3, 2F5, 3H8) produced modest inhibition (30–50%). The mmAb 2F5 was the weakest inhibitor, and the partial inhibition observed was not due to insufficient antibody since increasing the

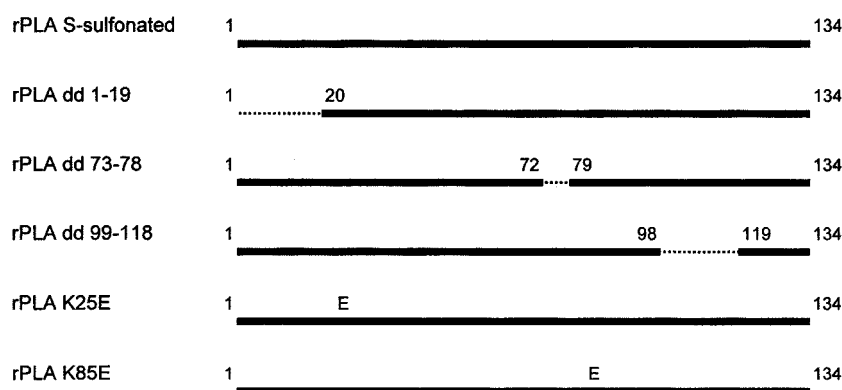


Figure 2. Variant rPLA molecules. The deleted segments are shown as dotted lines.

Table 2. Binding of hmAbs and mmAbs to solid phase bound PLA preparations^a

Group	Antibody	PLA native	rPLA dd 1–19	rPLA dd 73–78	rPLA dd 99–118	rPLA K25E	rPLA K85E	PLA acylated
I	BVA1	100	4	8	100	4	93	2
	BVA2	100	5	10	100	4	88	3
	830716	100	3	6	100	44	94	2
II	5D5	100	93	2	9	64	96	75
III	3F3	100	100	95	3	100	95	2
	1D12	100	100	91	3	100	100	2
	3H8	100	100	95	3	100	97	2
	1C11	100	100	92	2	98	92	2
	2B6	100	100	97	2	100	100	2
IV	8307175	100	100	100	2	100	100	87
	2F5	100	100	100	7	99	92	81
	1B76	100	100	98	63	100	100	93
V	831108	100	6	2	7	2	5	2
	831115	100	2	2	18	2	9	2

^a The binding of hmAbs and mmAbs to solid phase bound PLA preparations is given as a percentage of the binding measured with solid-phase bound native PLA.

antibody/antigen ratio from 10:1 to 20:1 did not increase the extent of inhibition. No inhibition was seen when boiled 2F5 was added to PLA or when all of the antibodies are incubated with porcine pancreatic phospholipase A₂ (data not shown), suggesting that the inhibition is due to PLA-antibody complexation.

Discussion

Two PLA-specific hAb and polyclonal antibodies of the IgG₄ isotype from 14 bee keepers who had prolonged and multiple exposure to PLA have previously been analysed in some detail and were shown to preferentially recognize the native three-dimensional structure of the molecule excluding the asparagine-linked sugar residue (Schneider *et al.*, 1994b). Further characterization of the epitopes recognized by these antibodies revealed that charged lysine residues on PLA were critical for antibody/antigen association (Schneider *et al.*, 1994a). Interestingly, the epitopes recognized by a significant proportion of the polyclonal serum IgG₄ antibodies from several beekeepers and both hmAbs contain lysine 25 as a contact residue (Dudler *et al.*, 1994), indicating a restricted epitope pattern.

Data from experiments described in this report support further the observation that both hmAbs only recognize correctly folded antigen, i.e. enzymatically active PLA (Table 2). None of the denatured or enzymatically inactive, truncated PLA molecules were recognized by the hmAbs. The only truncated PLA that is recognized by the two hmAbs is rPLA dd 99–118 that lacks a C-terminal surface loop and retains full enzymatic activity. This observation is consistent with the hmAb binding to the K25 epitope (Dudler *et al.*, 1994), which is located on the opposite side of the molecular surface.

Of all the mmAbs, the epitope recognition pattern of 830716 is the most similar to those of the two hmAbs. The only difference is that the binding of mmAb 830716 to rPLA K25E was about 40% of that measured with wild-type PLA, whereas both hmAbs failed to bind to rPLA K25E

(Table 2 and Dudler *et al.*, 1994). This difference may be the reason why the binding of either hmAb to PLA is not prevented by the complexation of mmAb 830716 to antigen (data not shown). In contrast, the binding of BVA1 and BVA2 to PLA is mutually exclusive (Schneider *et al.*, 1994b). These results indicate that the precise epitope recognized by mmAb 830716 is different from those of the hmAbs despite an apparent similar location of the epitopes. A further difference between the hmAbs and mmAb 830716 was apparent when these antibodies were tested for their capacity to inhibit the enzymatic activity of PLA (Table 3); mmAb 830716 caused complete inhibition, whereas the hmAbs inhibited the lipolysis reaction by 70–79%.

In contrast to both hmAbs, six of the 12 mmAbs recognized denatured, S-sulphonated PLA and native PLA to similar extents as measured by binding ELISA (Table 1). Inhibition ELISA experiments indicate that native PLA was

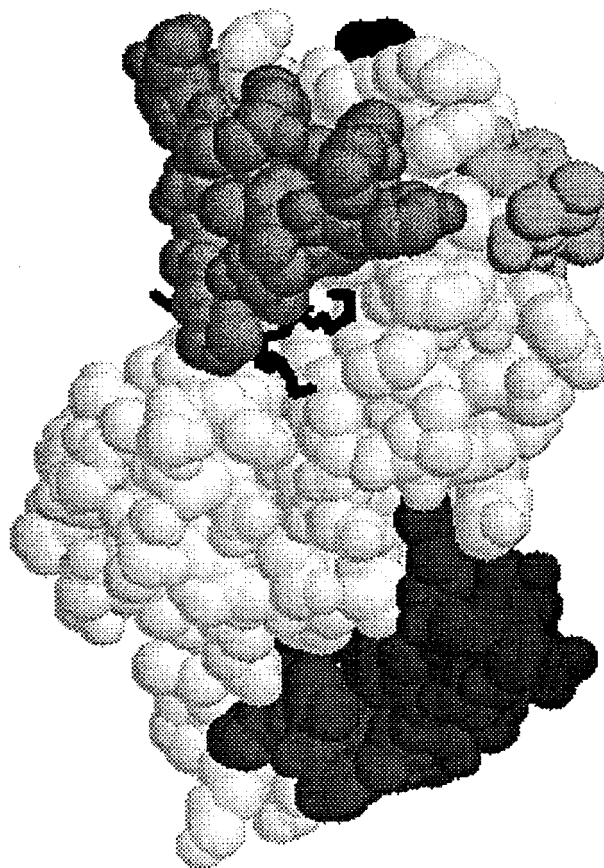


Figure 3. Localization of the epitopes on the X-ray structure of PLA recognized by PLA-specific mmAbs and hmAbs. Amino acids 1–19 (dark grey, top of the molecule); amino acid 25: (dark spot on top of the molecule); amino acids 73–76 (light grey at the top third of the PLA molecule at the right hand side); amino acids 99–118 (dark grey at the bottom of the molecule). The centre of the PLA is shown as light grey. The catalytic site slot is indicated by a y-shaped stick. Group I: discontinuous epitopes involving amino acids around positions 25 are bound by BVA1, BVA2 and 830716. Group II: epitope located in the centre of the polypeptide chain is recognized by 5D5. Groups III and IV: include at least four distinct epitopes near the C-terminal end; they are recognized by 3F3, 1D12, 3H8, 1C11 and 2B6 whose epitope(s) involve(s) one or more lysine residue; the same region is also recognized by 2F5, 1B76 or 8307175, respectively. Group V: epitopes including the carbohydrate part and N-terminal amino acids are recognized by 831115 and 831108.

Table 3. Effect of the antibodies on the enzymatic activity of native PLA

Group	Antibody	% Inhibition with antibody/PLA ratio ^a			
		20:1	10:1	5:1	2:1
I	BVA1 [12]		70	69	
	BVA2 [12]		79	75	n.d.
	830716		100	n.d.	100
II	5D5		63	60	
III	3F3		52	55	
	1D12		70	65	
	3HB		48	50	
	IC11		70	68	
	2B6		66	66	
IV	8307175		73		
	2F5 31 (boiled 4)		38		
	IB76		100	81	72
V	831108		100		66
	831115	79	77	77	100
Control ^b	R11683	< 10	< 10		

^a The antibody/PLA ratios are molar ratios.

^b The control serum (R11683) is a rabbit antiserum to the 85-kDa cytosolic PLA2 (Glover, S. de Carvalho, M.S., Bayburt, T., Jonas, M., Chi, E., Leslie, C. C. Gelb, M. H. JBC 270, 15359–367).

able to inhibit the binding of these six mmAbs to fluid phase-bound, native PLA by 6 to 90%.

This result shows that some of these six mmAbs recognize both denatured and native PLA and some recognize only denatured antigen. Presumably, this difference in PLA recognition is due to the possibility that some of the epitopes of these six mmAbs are cryptic in the context of the folded antigen. The finding that many of the isolated mmAbs recognize denatured PLA may be due to the fact that all mmAbs were selected by solid-phase screening. In general, proteins adsorbed on solid supports are partially denatured, and the location and nature of the denaturation are usually not known and cannot be predicted from the primary amino acid sequence or the X-ray structure of the antigen (Suter *et al.*, 1989; Butler *et al.*, 1992).

Two mmAbs recognize at least part of the asparagine-linked sugar residue of PLA. Recognition of sugar residues by IgG₄ antibodies is a rare event but is occasionally seen in serum from allergic individuals (Schneider *et al.*, 1994b).

It has recently been shown that the lipolytic activity of PLA is required for the induction of a strong IgE response to this allergen in mice (T. Dudler *et al.*, 1995). In addition, enzymatically active PLA, but not an inactive point mutant, can trigger mast cells to release a number of inflammatory mediators as well as the cytokine IL-4 (T. Dudler *et al.*, 1995). Thus, the ability of PLA-specific antibodies to inhibit its enzymatic function is of interest. The X-ray structure of

PLA shows that the catalytic site is a slot that extends through the enzyme about 15 Å from the protein's surface. The catalytic residues are tucked away in this slot and are not expected to be available for interaction with antibodies. Thus inhibition of the catalytic activity of PLA by antibodies is probably the result of the antibody interacting with the surface of the enzyme that is thought to contact the membrane interface. The surface, called the i-face (Ramirez and Jain, 1991) surrounds the catalytic site slot (Scott *et al.*, 1990b).

The inhibition data (Table 3) show that, in general, those antibodies that interact better with denatured PLA than with native PLA (group III) produce less inhibition of the enzymatic activity of PLA than those antibodies that recognize only the native protein. The hmAbs do not completely abolish the catalytic activity of PLA. The reason for this is not clear but it may be due to the fact that the epitope involving lysine 25 is not located on the i-face but rather off to the side. Group V mmAbs recognize the carbohydrate portion of PLA. The carbohydrate is on the edge of the i-face and this may explain why these mmAbs are potent PLA inhibitors. The mmAb 2F5 is the weakest inhibitor of PLA activity. At least one part of its epitope lies in the amino acid sequence 99–118 and much of this sequence is away from the i-face.

In summary, it is concluded that the antibodies induced by natural exposure of beekeepers to PLA have a restricted epitope recognition pattern in the context of an antigen with native-like structure. In contrast, mmAbs are heterogeneous in their epitope recognition patterns, although they can be organized into pattern recognition groups. Half of the mmAbs recognize denatured antigen and some of these fail to bind to native PLA. None of the 12 mmAbs was able to inhibit the binding of either hmAb to solid phase-bound PLA (Schneider *et al.*, 1994b and data not shown). In short, the use of mmAbs, obtained by artificial immunization of mice with antigen, for mapping the B cell epitopes recognized by antibodies in humans that have been naturally exposed to the same antigen has to be analysed with care.

Acknowledgements

We thank Dr W.D. Müller (Institut für Klinische Immunologie, Friedrich-Schiller-Universität Jena, Germany) for providing PLA-specific murine monoclonal antibodies. This work was supported by the Swiss National Science Foundation Grant NSF 31-28815.90 and by a grant from the National Institutes of Health (HL-36235). M.H.G. is the recipient of a National Institutes of Health Research Career Development Award (GM-562). R.R.A. is the recipient of a National Institutes of Health postdoctoral fellowship (GM-15464).

References

- Alexander, H., Alexander, S., Getzoff, E. D., Tainer, J. A., Geysen, H. M. and Lerner, R. A. (1992). Altering the antigenicity of proteins. *Proc. Natl. Acad. Sci. USA* **89**, 3352.
- Bahraoui, E., Yagello, M., Billaud, J.-N., Sabatier, J.-M., Guy, B., Muchmore, E., Girard, M. and Gluckman, J.-C. (1990). Immunogenicity of the human immunodeficiency virus (HIV) recombinant nef gene product. Mapping of T-cell and B-cell epitopes in immunized chimpanzees. *AIDS Res. Hum. Retroviruses* **6**, 1087.
- Berzofsky, J. A. (1985). Intrinsic and extrinsic factors in protein antigenic structure. *Science* **229**, 932.
- Bousquet, J., Fontez, A., Aznar, R., Robinet-Levy, M., and Michel, F.-B. (1987). Combination of passive and active immunization in honeybee venom immunotherapy. *J. Allergy Clin. Immunol.* **79**, 947.
- Boyer, V., Broly, H., Souche, S., Madaule, P., Rossier, J., Zagury, D. and Desgranges, C. (1991). Characterization and large production of human monoclonal antibodies against the

- HIV-1 envelope. *Clin. Exp. Immunol.* **83**, 452.
- Butler, J. E., Ni, L., Nessler, R., Joshi, K. S., Suter, M., Rosenberg, B., Chang, J., Brown, W. R. and Cantarero, L. A. (1992). The physical and functional behavior of capture antibodies adsorbed on polystyrene. *J. Immunol. Methods* **150**, 77.
- Cunningham, B. C., Jhurani, P., Ng, P. and Wells, J. A. (1989). Receptor and antibody epitopes in human growth hormone identified by homolog-scanning mutagenesis. *Science* **243**, 1330.
- Dudler, T., Chen, W.-Q., Wang, S., Schneider, T., Annand, R. R., Dempcy, R. O., Cramer, R., Gmachl, M., Suter, M. and Gelb, M. H. (1992). High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A₂. *Biochim. Biophys. Acta* **1165**, 201.
- Dudler, T., Machado, D. C., Kolbe, L., Annand, R. R., Rhodes, N., Gelb, M. H., Koelsch, K., Suter, M. and Helm, B. A. (1995). A link between catalytic activity, IgE-independent mast cell activation, and allergenicity of bee venom phospholipase A₂. *J. Immunol.* **155**, 2605–13.
- Dudler, T., Schneider, T., Annand, R. R., Gelb, M. H. and Suter, M. (1994). The antigenic surface of the bee venom allergen phospholipase A₂: structural and functional analysis of human IgG4 antibodies reveals potential role in protection. *J. Immunol.* **152**, 5514.
- Getzoff, E. D., Geysen, H. M., Rodda, S. J., Alexander, H., Tainer, J. A. and Lerner, R. A. (1987). Mechanisms of antibody binding to a protein. *Science* **235**, 1191.
- Geysen, H. M., Rodda, S. J., Mason, T. J., Tribbick, G., and Schoffs, P. G. (1987a). Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods* **102**, 259.
- Geysen, H. M., Tainer, J. A., Rodda, S. J., Mason, T. J., Alexander, H., Getzoff, E. D. and Lerner, R. A. (1987b). Chemistry of antibody binding to a protein. *Science* **235**, 1184.
- Ho, D. D., Fung, M. S. C., Cao, Y., Li, X. L., Sun, C., Chang, T. W. and Sun, N.-C. (1991). Another discontinuous epitope on glycoprotein gp120 that is important in human immunodeficiency virus type 1 neutralization is identified by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA* **88**, 8949.
- Hopp, T. P. (1986). Protein surface analysis. Methods for identifying antigenic determinants and other interaction sites. *J. Immunol. Methods* **88**, 1.
- Jain, M. K. and Gelb, M. H. (1991). Phospholipase A₂-catalyzed hydrolysis of vesicles: uses of interfacial catalysis in the scooting mode. *Methods Enzymol.* **197**, 112.
- Jin, L., Fendly, B. M. and Wells, J. A. (1992). High resolution functional analysis of antibody-antigen interactions. *J. Mol. Biol.* **226**, 851.
- Kuchler, K., Gmachl, M., Sippl, M. J. and Kreil, G. (1989). Analysis of the cDNA for phospholipase A₂ from honeybee venom glands. The deduced amino acid sequence reveals homology to the corresponding vertebrate enzymes. *Eur. J. Biochem.* **184**, 249.
- Lessof, M. H., Sobotka, A. K. and Lichtenstein, L. M. (1978). Effects of passive antibody in bee venom anaphylaxis. *The Johns Hopkins Medical Journal* **142**, 1.
- Martin, D., Munson, R., Jr., Grass, S., Chong, P., Hamel, J., Zobrist, G., Klein, M. and Brodeur, B. R. (1991). Mapping of B-cell epitopes on the outer membrane P2 porin protein of *Haemophilus influenzae* by using recombinant proteins and synthetic peptides. *Infect. Immun.* **59**, 1457.
- Müller, U. R., Morris, T., Bischof, M., Friedli, H. and Skarvil, F. (1986). Combined active and passive immunotherapy in honeybee-sting allergy. *J. Allergy Clin. Immunol.* **78**, 115.
- Müller, W.-D., Fahlbusch, B., Gebhardt, M., Jung, K., and Jäger, L. (1993). Characterization of murine monoclonal antibodies to phospholipase A₂ and mellitin from bee venom. *Int. Arch. Allergy Immunol.* **101**, 153.
- Ohara, N., Matsuo, K., Yamaguchi, R., Yamazaki, A., Tasaka, H. and Yamada, T. (1993). Cloning and sequencing of the gene for α antigen from *Mycobacterium avium* and mapping of B-cell epitopes. *Infect. Immun.* **61**, 1173.
- Ramirez, F. and Jain, M. K. (1991). Phospholipase A₂ at the bilayer interface. *Proteins* **9**, 229.
- Schneider, T., Lang, A. B., Carballido, J. M., Santamaria Babi, L. F., Dudler, T., Kägi, M. K., Blaser, K. and Suter, M. (1994a). Human monoclonal or polyclonal antibodies recognize predominantly discontinuous epitopes on bee venom phospholipase A₂. *J. Allergy Clin. Immunol.* **94**, 61.
- Schneider, T., Dudler, T., Gelb, M. H. and Suter, M. (1994b). Lysine residues in bee venom phospholipase A₂ are important for binding to human monoclonal or polyclonal antibodies of the IgG4 isotype. *Int. Arch. Allergy Immunol.* **104**, 262.
- Scott, D. L., Otwinowski, Z., Gelb, M. H. and Sigler, P. B. (1990a). Crystal structure of bee-venom phospholipase A₂ in a complex with a transition-state analogue. *Science* **250**, 1563.
- Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H. and Sigler, P. B. (1990b). Interfacial catalysis: the mechanism of phospholipase A₂. *Science* **250**, 1541.
- Smith, T. J., Olson, N. H., Holland Cheng, R., Liu, H., Chase, E. S., Ming Lee, W., Leippe, D. M., Mosser, A. G., Rueckert, R. R. and Baker, T. S. (1993). Structure of human rhinovirus complexed with Fab fragments from a neutralizing antibody. *J. Virol.* **67**, 1148.
- Söderland, E., Lagerkvist, A. C. S., Dueñas, M., Malmberg, A.-C., Ayala, M., Danielsson, L., and Borrebaeck, C. A. K. (1993). Chaperonin assisted phage display of antibody fragments on filamentous bacteriophages. *Bio/Technology* **11**, 503.
- Stanfield, R. L., Fieser, T. M., Lerner, R. A. and Wilson, I. A. (1990). Crystal structures of an antibody to a peptide and its complex with peptide antigen at 2.8 Å. *Science* **248**, 712.
- Strasburger, C. J., Kostyo, J., Vogel, T., Barnard, G. J. and Kohen, R. (1989). The antigenic epitopes of human growth hormone as mapped by monoclonal antibodies. *Endocrinology* **124**, 1548.
- Stüber, D., Bannwarth, W., Pink, J. R. L., Meloen, R. H. and Matile, H. (1990a). New B cell epitopes in the *Plasmodium falciparum* malaria circumsporozoite protein. *Eur. J. Immunol.* **20**, 819.
- Stüber, D., Matile, H. and Garotta, G. (1990b). System for high level production in *E. coli* and rapid purification of recombinant proteins: applications to epitope mapping, preparation of antibodies and structure-function analysis. In *Immunological Methods, Volume IV*, ed. by I. Lefkowitz and B. Pernis, p. 121. Academic Press, New York.
- Suter, M., Butler, J. E. and Peterman, J. H. (1989). The immunochemistry of sandwich ELISAs. III. The stoichiometry and efficacy of the protein-avidin-biotin capture (PABC) system. *Mol. Immunol.* **26**, 221.
- Yuan, J. and Low, P. S. (1992). Epitope mapping by a method that requires no amino acid sequence information. *Anal. Biochem.* **205**, 179.
- Yuan, W., Quinn, D. M., Sigler, P. B. and Gelb, M. H. (1990). Kinetic and inhibition studies of phospholipase A₂ with short-chain substrates and inhibitors. *Biochemistry* **29**, 6082.
- Zhang, L., Olsen, E., Kisil, F. T., Hill, R. D., Sehon, A. H. and Mohapatra, S. S. (1992). Mapping of antibody binding epitopes of a recombinant *Poa p* IX allergen. *Mol. Immunol.* **29**, 1383.
- Zinn-Justin, S., Roumestand, C., Drevet, P., Ménez, A. and Toma, F. (1993). Mapping of two "neutralizing" epitopes of a snake curaremimetic toxin by proton nuclear magnetic resonance spectroscopy. *Biochemistry* **32**, 6884.