

Modulation of the Immune Response to Allergens: Phospholipase A Degradation Products Suppress IgG and IgE Response in Mice

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Abstract. Degradation products of phospholipase A₂ (PLA₂), the major allergen of honeybee venom, were prepared by peptic digestion of the enzyme and fractionated by molecular exclusion chromatography. The largest of the isolated products, P-1, was found to be a centrally nicked PLA₂ molecule with a loss of several amino acids. It was examined for its antigenic, immunogenic and immunosuppressive properties. This peptic product was unable to block antigen-antibody reactions between PLA₂ and anti-PLA₂ antibodies and was incapable of inducing passive cutaneous anaphylaxis in rat skin sensitized with mouse anti-PLA₂ IgE antibodies. BDF₁ female mice injected intravenously with P-1 exhibited suppressed IgG and IgE anti-PLA₂ responses to a subsequent PLA₂ immunization. Intravenous administration of P-1 to mice with ongoing immune response to PLA₂ resulted in decline of antigen-specific IgE titers. Transfer of splenic T cells from mice injected with P-1 into naive recipients caused profound suppression of the response to subsequent immunization with PLA₂ indicating presence of PLA₂-specific T suppressor cells. These results indicate that a degradation product of PLA₂ prepared by peptic digestion lost antigenic and immunogenic properties of the intact enzyme but retained immunosuppressive properties, thus offering a novel approach to allergen immunotherapy.

Introduction

We have demonstrated that it is possible to significantly reduce an immune response to a complex protein antigen, bovine serum albumin (BSA), by pretreatment of mice with polypeptides obtained by peptic digestion of this antigen. These BSA fragments were poorly immunogenic. However, intravenous injection of such fragments into mice suppressed the response to a subsequent BSA challenge. This suppression was mediated by T cells [1]. In this study, we attempted to obtain similar immunoregulatory effects with degraded polypeptide products from an enzyme involved in human immediate hypersensitivity disease. Honeybee venom phospholipase A₂ (PLA₂) is the major allergen present in honeybee venom, and is responsible for causing anaphylaxis in humans to honeybee stings [2]. This allergen was selected because it is relatively easily prepared in pure form from honeybee venom and because the clinical disease caused by it is common enough to allow application of animal experimental data to humans. In the experiments described herein, we explored a minimally degraded

peptic digestion product of PLA₂, P-1. This product was characterized as to its antigenicity, immunogenicity, reactivity with anti-PLA₂-specific IgE and IgG antibodies, and its ability to suppress primary and ongoing immune responses to PLA₂.

Materials and Methods

Mice

BDF₁ female mice, 9-12 weeks old, were purchased from Jackson Laboratory, Bar Harbor, Me. Each experimental group consisted of 5 animals and each experiment was repeated 3 times.

Reagents

Honeybee venom was donated by Champlain Valley Apiaries (Middlebury, Vt.). Sephadex G-50 was purchased from Pharmacia, Piscataway, N.J., and pepsin from Sigma Chemical Co., St. Louis, Mo.

Purification of PLA₂ from Bee Venom (fig. 1)

PLA₂ was purified following the method of Shipolini et al. [3, 4]. Lyophilized honeybee venom (0.5 g) was dissolved in 7 ml of 0.05 M ammonium acetate, pH 4.75, and clarified by centrifugation for 10 min at 10,000 g. The supernatant was then applied onto a 2.5 × 192 cm Sephadex G-50 (superfine) column equilibrated with

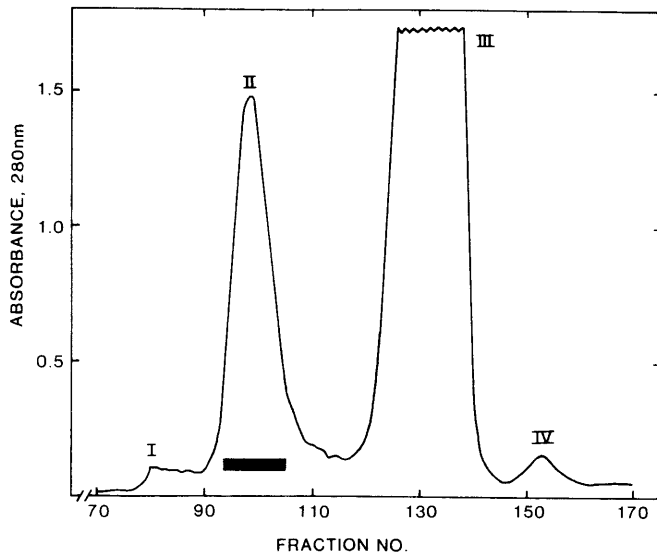


Fig. 1. Gel filtration of the soluble fraction of crude bee venom on Sephadex G-50 column (fine). Bar indicates fractions pooled.

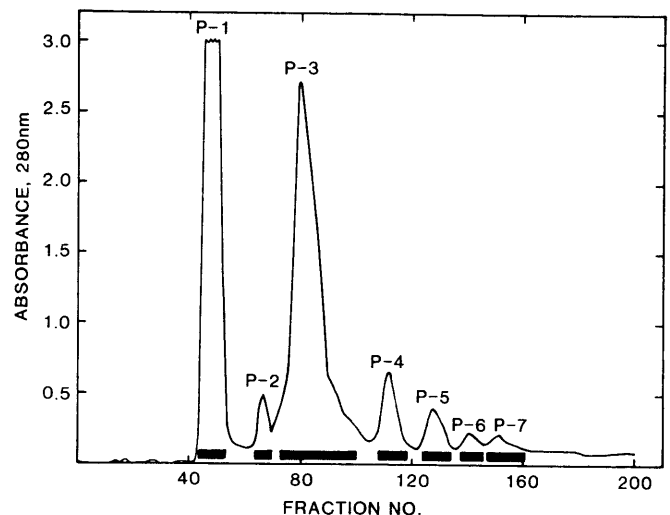


Fig. 2. Effluent profile of the peptic digest of native phospholipase A₂ following gel filtration on sephadex G-25. The column (1.5 × 93 cm) was equilibrated with 5% formic acid. Fractions of 1.4 ml were collected at a flow rate of 15 ml/h. Bars indicate pooled fractions.

the same buffer. Fractions of 5.8 ml were collected at a flow rate of 87 ml/h. The second fraction containing all the PLA₂ activity was lyophilized.

Digestion of PLA₂ with Pepsin (fig. 2)

PLA₂ (97 mg) was dissolved in 10 ml 5% formic acid and 2 mg pepsin added. The digestion was carried out at 25°C for 6 h. Proteolysis was terminated by the addition of 1 N NaOH, raising the pH to 8.9, which destroyed any residual pepsin activity. The digested products of PLA₂ were separated into individual fractions by Sephadex G-50 (superfine) chromatography. The first eluting product peak was termed P-1. This peak was concentrated and lyophilized.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The method of Weber and Osborne [5] was employed.

Measurement of Phospholipase Activity

Phospholipase activity was measured by a modification of the method of Cottrell [6]. Two hundred fifty milligrams of dimyristoyl phosphatidylcholine were dissolved in 25 ml of n-propanol yielding a 10 mg/ml solution. Nine milliliters of this solution were mixed with 3 ml of a solution of 500 μ M CaCl₂ and 50 μ M EDTA. The pH of the resultant solution was adjusted to 8.0 with 0.01 N NaOH in n-propanol (1:3 v/v). Lipolysis was carried out by addition of 50 μ l of 10 mg/ml solution of PLA₂ or its peptic product, P-1. The pH was kept constant by further addition of 0.01 N NaOH in n-propanol. The phospholipase activity of P-1 was compared to intact PLA₂ based on the amount of NaOH required to keep the pH constant.

Passive Cutaneous Anaphylaxis

Doubling dilutions of pooled sera (100 μ l) from 5 mice were injected intradermally 2 cm apart on the shaved skin of anesthetized Sprague-Dawley adult male rats. After 24 h, 0.01–0.05 mg of PLA₂ mixed with 0.5% Evans blue was injected through the penile vein of the rats. Reaction sites on the skin were measured after 20 min. The titer was expressed as the reciprocal value of the highest dilution giving a positive reaction of at least 5 mm in diameter. A variation of > 2 fold was found to be statistically significant [7]. Heating of sera at 56°C for 2 h eliminated their ability to induce passive cutaneous anaphylaxis (PCA), thus validating that PCA reaction was IgE-specific. Each serum was tested in 3 rats.

IgG Antibody Assay

A quantitative ELISA technique was used as described [8]. Standard curves with an affinity-purified mouse IgG preparation were run each time an assay was performed. The ELISA results are expressed in EU units with 1 EU equivalent to 1 μ g of the standard. We employed several concentrations of antigens as inhibitors which were incubated overnight at 4°C with a constant dilution of antiserum. The mixtures were then assayed on PLA₂-coated ELISA plates. Pooled sera were collected from groups of 5 mice. Each experiment was repeated at least 2 times. Significance of the results was determined by Student's t test.

Pretreatment and Immunization

Fraction P-1 was dissolved in saline and administered intravenously. Animals were immunized with PLA₂ intraperitoneally with aluminum hydroxide gel (alum) as adjuvant (2 mg/injection).

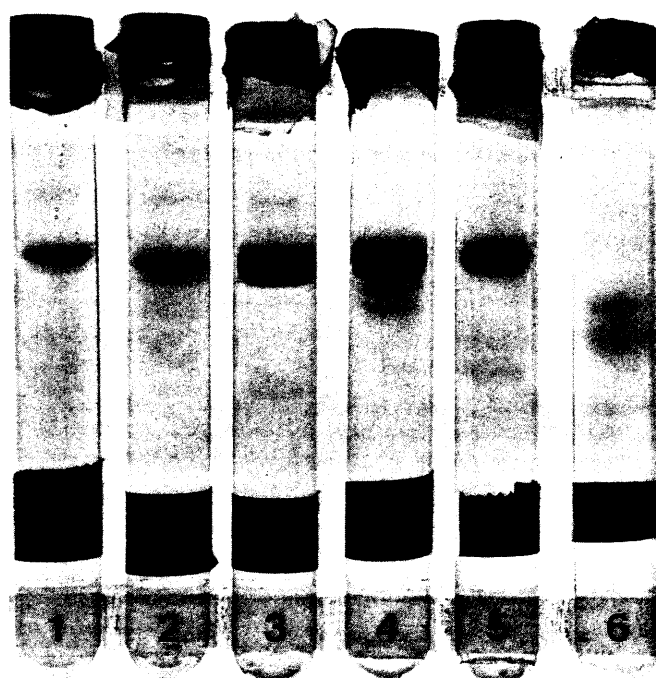


Fig. 3. The SDS gel electrophoresis patterns of phospholipase A_2 and P-1. From left to right: gel 1, PLA_2 from Sigma Chemical Co.; gel 2, PLA_2 with 1.0% β -mercaptoethanol; gel 3, PLA_2 prepared according to text; gel 4, PLA_2 with 1.0% β -mercaptoethanol; gel 5, P-1; gel 6, P-1 with 1.0% β -mercaptoethanol.

Cell Transfers

Single cell splenic suspensions were prepared in cold Hanks' balanced salt solution. Enrichment for T cells was done by passing the cell suspension over a nylon wool column [9]. The yield of the effluent (T cell-enriched) fraction was 20–25% of the cells applied to the column. Treatment with anti-thy 1.2 antiserum and complement (Accurate Chemical and Scientific Corp., Westbury, N.Y.) resulted in the killing of 95% of the cells as judged by trypan blue exclusion. The nylon wool-adherent cells were resuspended in complete RPMI 1640 medium containing 10% fetal calf serum.

Cell transfers were performed by intravenous injection of 10^7 T-enriched splenic cells to naive syngeneic mice; the recipients were immunized immediately with PLA_2 .

Results

Fragment Preparation and Characterization

Seven protein fractions were obtained from digestion. The first major component, fraction P-1, was used in this study to modulate the immune response. Fraction P-1 had an elution volume indistinguishable from the undigested molecule but showed no phospholipase activity. The fraction P-1 was examined on

Table 1. P-1 and PLA_2 immunogenicity in BDF₁ mice

Immunizing antigen injected i.p. ¹	Anti- PLA_2 response			
	ELISA IgG titer EU/ml		IgE titer	
	14 days	21 days	14 days	21 days
P-1/100 μ g	nil	nil	nil	nil
P-1/10 μ g	nil	nil	nil	nil
P-1/1 μ g	nil	nil	nil	nil
PLA_2 /10 μ g	650 ± 27	$1,210 \pm 50$	320	320
PLA_2 /1 μ g	440 ± 20	680 ± 25	160	160

¹ $Al(OH)_3$ as adjuvant, 1 mg/mouse.

Table 2. Antigenicity of P-1 fragment

Antigen tested	Minimal amount of antigen needed to inhibit ELISA IgG μ g/ml	Minimal amount of antigen needed to induce PCA reaction μ g/ml
PLA_2	20	10
P-1	> 500	> 1,000

sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and found to have a molecular size similar to that of the native molecule (fig. 3). Amino acid analysis (kindly performed by Dr. T. Peters, Cooperstown, N.Y.) showed a loss of approximately 8 amino acid residues and comparison with the known amino acid sequence of PLA_2 [10] led to the conclusion that the missing residues were located between positions 75 and 82. This assumption was further supported by electrophoresis on SDS polyacrylamide gel (fig. 3). After incubation of P-1 with β -mercaptoethanol, P-1 separated into two fragments of similar size, which lend further validity to the thesis that P-1 is a nicked version of the PLA_2 molecule with a deletion in the central portion.

Immunogenicity and Antigenicity (tables 1, 2)

The immunogenicity of P-1 was tested in mice with alum as adjuvant. As measured by ELISA for IgG antibodies and PCA assay for IgE antibodies, P-1 was found to be nonimmunogenic at a dose of 100 μ g/mouse. In contrast, untreated PLA_2 was immunogenic

Table 3. Suppression of primary response to PLA₂ by P-1 in BDF₁ mice

Pretreatment (day 9, 8, 7) i.v.	Immunization (day 0) i.p. ¹	Anti-PLA ₂ response			
		ELISA IgG titer, EU/ml		IgE titer	
		14 days	21 days	14 days	21 days
P-1/10 µg	PLA ₂ /10 µg	65 ± 10*	80 ± 10*	20* (10-20)	40* (20-40)
P-1/10 µg	-	nil	nil	nil	nil
-	PLA ₂ /10 µg	440 ± 50	620 ± 30	640 (640-1,280)	640
P-1/10 µg	OVA/10 µg	2,500 ± 120	3,200 ± 70	320 (320-640)	160 (80-160)
-	OVA/10 µg	2,350 ± 80	3,400 ± 160	320	160 (80-160)

* $p < 0.001$ (Student's *t* test), compared to a control group that was not pretreated with P-1.

¹ OVA = Hen egg albumin. Al(OH)₃ as adjuvant, 1 mg/mouse.

at a dose of 1 µg/mouse. Sera were also tested on ELISA plates coated with P-1 to test for antibody unique to the product. Results were uniformly negative.

P-1 was tested for its ability to inhibit PLA₂-anti-PLA₂ IgG and IgE antigen-antibody reactions. Also the ability of P-1 to evoke a PCA reaction in the skin of rats presensitized with mouse anti-PLA₂ IgE antibodies was tested. P-1 was unable to block either IgG or IgE PLA₂-anti-PLA₂ reactions. Furthermore, no PCA reactions were observed when the rats sensitized with anti-PLA₂ serum were challenged with as much as 1,000 µg P-1. In contrast rats sensitized with anti-PLA₂ serum were distinctly positive when challenged with 10 µg of intact PLA₂.

We conclude that P-1 lacks major B cell determinant(s) of PLA₂ and this loss of major B determinants is responsible for the loss of antigenicity and immunogenicity.

Immunosuppression (tables 3, 4)

The ability of P-1 to regulate the immune response to PLA₂ was tested using a pretreatment regimen of 10 µg given intravenously for 3 days prior to challenge with PLA₂. The immune response to PLA₂ was significantly suppressed as measured by ELISA and PCA. The PCA titer was reduced 32-fold by this pretreatment regimen and the IgG response 4-fold. Pretreatment with P-1 had no effect on immune responsiveness to an unrelated antigen, hen egg albumin.

The ability of P-1 to immunosuppress an ongoing immune response was tested by intravenous adminis-

Table 4. Suppression of ongoing anti-PLA₂ response with P-1 in BDF₁ mice

Day 0 immunized	Day 30 IgE titer	Day 60 IgE titer	Days 61-66 treated	Day 75 IgE titer
PLA ₂ 10 µg	80 (80-160)	80 (80-160)	P-1 100 µg	20* (10-20)
PLA ₂ 10 µg	80 (80-160)	80 (40-160)	nil	80

Animals were immunized i.p. with PLA₂ in Al(OH)₃ (1 mg) on day 0 and the immune response measured on days 30, 60 and 75. The treated group was given 100 µg P-1 i.v. on days 61-66.

* $p < 0.04$ (Student's *t* test), compared to a group not treated with P-1.

Table 5. T cell transfer from P-1-treated mice to naive recipients

T cells (1×10^7) transferred from animals with the following treatment	Immunization with PLA ₂ (10 µg) ^a	Anti-PLA ₂ response ELISA IgG titer, EU/ml	
		14 days	21 days
3 × 100 µg P-1 i.v.	+	70 ± 10*	80 ± 15*
3 × 100 µg P-1 i.v.	-	nil	nil
Saline	+	470 ± 25	550 ± 40
Saline	-	20 ± 5	20 ± 5
No T cell transfer	+	520 ± 30	660 ± 50

* $p < 0.01$ (Student's *t* test), compared with a saline-treated, PLA₂-immunized group.

^a Al(OH)₃ as adjuvant, 1 mg/mouse.

tration of P-1 on days 61–65 postimmunization to animals immunized with PLA₂. This treatment resulted in a reduction in IgE antibody titers.

Mechanism of Suppression (table 5)

To determine the mechanism of P-1-induced suppression, groups of mice were pretreated intravenously with P-1 for 3 successive days. One week later the animals were sacrificed and splenic T cell suspensions were prepared. T cells were transferred into naive syngeneic mice which were then immunized intraperitoneally with PLA₂ in alum. The 5 mice which had been given splenic T cells from P-1-treated animals were immunosuppressed indicating the presence of PLA₂-specific T suppressor cells.

Discussion

Several previous investigations have reported that chemical modifications of allergens can enhance their immunosuppressive properties [11–13]. These modifications involved denaturation or conjugation of the allergens with polyethylene glycol or immunoglobulins. In these preparations, molecular size of the allergen was not reduced. Enzymatic digestion of allergens results in degradation of the polypeptide backbone of the molecule with consequent elimination or modification of B cell determinants. The topography of the major determinants consists of residues originating from different parts of the antigen molecule. Deletion of only a few critical amino acid residues can lead to loss of major B cell determinants. For example, in the case of the pigeon cytochrome C molecule, deletion of residues 100–104 would be expected to result in loss of antibody reactivity with the antigen [14].

This study extends our earlier investigations with BSA in which we discovered that fragments derived by peptic digestion of BSA have the capacity to downregulate the immune response [1] while being relatively nonimmunogenic [15]. We showed that this immunoregulation could be attributed to the activation of suppressor T lymphocytes. In the present study we utilized a well-defined human allergen, PLA₂, which is the major allergenic component of honeybee venom. This allergen was enzymatically digested with pepsin and a major degradation product, P-1, was isolated and subsequently tested for immunoregulatory properties. The P-1 molecule exhibited

no enzymatic activity and was neither antigenic, nor immunogenic. Thus, similar to our studies with BSA the major B cell-specific antigenic determinant(s) was eliminated by the peptic digestion. However, the immunosuppressive properties of P-1 seemed to be unaffected, suggesting that the suppressive T cell determinants were preserved. One of the limiting factors in the use of unaltered protein allergens in allergen immunotherapy is the retention of B determinants which causes allergic reactions to occur due to binding and cross-linking of specific IgE on the mast cells. This, in turn, limits the quantity of intact allergen that can be administered safely. As high-dose immunotherapy has been shown to be considerably more effective than low-dose treatment, this dosage restriction may limit the 'favorable' stimulatory effect on T cells by the conventional immunotherapy [16]. Limited pepsin degradation of phospholipase A₂ is an attempt to bypass this problem.

In a recent clinical study [17] patients with anaphylactic sensitivity to honeybee venom were treated with P-1. This preparation caused only rare reactions with doses up to 100 µg/injection. Treatment resulted in a substantial decrease in specific anti-PLA₂ IgG and IgE antibodies as well as a decline in skin test sensitivity to PLA₂. Thus, administration of antigenic fragments in both humans and mice had similar immunoregulatory effects. However, at this time it has not been established whether the mechanism of immunoregulation in humans and animals are the same. Nevertheless, our data demonstrate that a nonimmunogenic product derived from an allergen can downregulate immune responses, and that this procedure offers a new modality for the therapy of allergic diseases.

Acknowledgments

We wish to thank Dr. Theodore Peters, Jr. for the SDS gel and amino acid analyses and Ms. Barbara Cromer for the preparation of the manuscript. This work was supported in part by a grant from NIH No. AI 15520.

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Received: February 21, 1990

Accepted after revision: May 14, 1990

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