

Type I skin reactivity to native and recombinant phospholipase A₂ from honeybee venom is similar

Ulrich R. Müller, MD,^a Thomas Dudler, PhD,^b Theres Schneider, PhD,^b
Reto Cramer, PhD,^b Hugo Fischer, MD,^a Dejan Skrbic, MD,^a
Rudolf Maibach, PhD,^a Kurt Blaser, PhD,^b and Mark Suter, MD^b
Bern and Davos, Switzerland

Phospholipase A₂ is the major allergen in honeybee venom. Recombinant phospholipase A₂ was produced in prokaryotes and tested for its biologic activity by intracutaneous skin testing with serial 10-fold dilutions in comparison with native and deglycosylated phospholipase A₂ in patients allergic to bee venom. Linear regressions of the log of the wheal area versus the log of the allergen concentration were calculated for all allergens in each patient. The relative allergenic potency of the various preparations was analyzed by comparing the linear regressions. Native phospholipase A₂ was about 10 times more potent than whole bee venom. None of 58 patients allergic to bee venom was missed by testing with native phospholipase A₂ alone. This allergen and deglycosylated native phospholipase A₂ resulted in similar skin reactions, indicating that the sugar residues were of little relevance for IgE-binding in the patients tested. Native phospholipase A₂ also had relative potency similar to that of recombinant refolded phospholipase A₂ whereas recombinant nonrefolded phospholipase A₂ had almost no biologic activity in skin testing. These results demonstrate in vivo activity of the recombinant bee venom allergen phospholipase A₂. Although correct refolding is a prerequisite for type I skin reactivity, glycosylation seems to be less important. (J ALLERGY CLIN IMMUNOL 1995;96:395-402.)

Key words: Skin reactivity, phospholipase A₂ honeybee venom, recombinant DNA technology

Recombinant DNA technology has been used successfully to clone and to elucidate the structure of genes encoding allergens from house dust mites,¹ grass pollen,² birch pollen,³ ragweed pollen,⁴ cat dander,⁵ *Dolichovespula* venom,⁶ and fungi.⁷ Analysis of these genes provides detailed knowledge about the primary structure of allergens.

Recent improvements in biotechnology allow the production of pure recombinant allergens in large amounts.^{7,8} In contrast to allergens purified by biochemical methods, recombinant proteins are

Abbreviations used

BV:	Honeybee venom
ICC ₁₀₀ :	Log of the concentration (in grams per liter) resulting in a 100 mm ² wheal in intracutaneous skin testing
PLA:	Phospholipase A ₂ from honeybee venom
PLA _d :	Deglycosylated phospholipase A ₂
PLA _n :	Native phospholipase A ₂
PLA _{nr} :	Recombinant nonrefolded phospholipase A ₂
PLA _r :	Recombinant refolded phospholipase A ₂

From ^athe Medical Division, Zieglerspital, Bern, and ^bthe Swiss Institute of Allergy and Asthma Research, Davos.

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Reprint requests: Ulrich R. Müller, MD, Medical Division, Zieglerspital, CH-3007 Bern, Switzerland.

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free of contaminating allergens from the same allergen source. Hence recombinant proteins promise to improve diagnostic precision in allergology substantially and may lead to a better understanding of the basic pathophysiologic mechanisms of IgE-mediated allergic diseases.

Hymenoptera stings, particularly those of the honeybee (*Apis mellifera*) and various vespids, are a well-known cause of IgE-mediated anaphylaxis.⁹ Such reactions may be life threatening and occasionally even fatal. Bee venom phospholipase A₂ (PLA) was identified as the major allergen of honeybee venom (BV) many years ago.¹⁰ PLA was also used to induce human secondary immune responses and antibody production in vitro.¹¹ Recently complementary DNA cloning of PLA was described¹² and recombinant PLA (PLA_r) expressed in prokaryotes is available.¹³ Its immunologic activity has been investigated in vitro.¹⁴ In this article we describe the in vivo activity of PLA_r in comparison with its native purified counterpart and to whole BV in skin testing of persons allergic to bee venom.

METHODS

Patients and control subjects

Fifty-eight adult patients with a well-defined history of allergic reactions to honeybee stings and positive diagnostic test reactions to BV were screened. Of the 17 who were finally selected for more detailed analysis, 16 had had a systemic allergic reaction and one had a large local reaction to honeybee stings. All had a positive intracutaneous skin test reaction to BV at a concentration of 10⁻⁴ gm/L (0.1 µg/ml) or less.^{15,16} BV-specific IgE serum antibodies were detected by RAST in 16 of the 17 patients. None of the patients was treated by BV immunotherapy before or at the time of testing. Twenty-two healthy adults with no history of allergic reactions to bee stings, a negative skin test reaction to BV at 10⁻³ gm/L (1 µg/ml), and a negative RAST result to BV served as control subjects.

The study protocol was approved by the ethical committee of the medical faculty of the University of Bern. All persons gave their informed consent to the skin tests with recombinant PLA.

Allergens

BV for skin testing was obtained from Pharmacia AB, Uppsala, Sweden. Native bee venom PLA (PLA_n) (Boehringer, Mannheim, Germany) was used directly as supplied or after deglycosylation. Allergen concentrations refer to their protein content. For deglycosylation 500 µg of PLA_n was dissolved in 1 ml of 250 mmol/L sodium acetate, pH 6.5, containing 1 mmol/L phenylmethylsulfonyl fluoride (Fluka, Buchs, Switzerland). To this 1 U of endoglycosidase F-N-glycosidase (Boehringer) was added and the mixture incubated for 24 hours at 37° C, followed by dialysis against phosphate-buffered saline solution (pH 7.4) for 3 days at room temperature. Complete deglycosylation was verified by polyacrylamide gel electrophoresis.¹⁷ Deglycosylated PLA (PLA_d) was stored at 4° C for no more than 1 week before use.

PLA_r was produced as described recently.¹³ Briefly, a synthetic gene encoding the mature form of PLA¹² was designed and subcloned in a prokaryotic high-level expression vector¹⁸ provided by Dr. D. Stüber (Hoffmann-La Roche, Basel, Switzerland). PLA_r was expressed, purified, and refolded as described¹³ and was used without removal of the [histidine]₆ affinity peptide. Biologic activity of all PLA preparations was determined by enzymatic assays¹⁹ and by antibody-binding with human monoclonal and polyclonal antibodies.¹⁴ In these tests no difference was found between PLA_n and PLA_r. For some tests sulfonated but nonrefolded recombinant PLA (PLA_{nr}) was used. In contrast to the refolded preparation, PLA_{nr} had neither enzymatic nor antigenic activity in vitro.¹³

The PLA content of the BV stock solution of 100 µg/ml was estimated in two ways: (1) by a spectrophotometric enzymatic assay²⁰ and (2) by inhibition ELISA with the human monoclonal antibody BVA₂.²¹ The PLA content found by the enzymatic assay was 16.4 µg/ml, and that by ELISA inhibition was 10 µg/ml.

Skin testing

For quantitative skin testing a stock solution of 10⁻¹ gm/L (100 µg/ml) of native or recombinant allergens was prepared in commercial skin test diluent (Pharmacia AB, Uppsala, Sweden), a phosphate buffer containing 0.03% human serum albumin. The preparations were sterilized by microfiltration (0.2 µm pores; Millipore, Bedford, Mass.) and stored at 4° C for not more than 1 week.

Serial 10-fold dilutions ranging from 10⁻² to 10⁻⁸ gm/L were then prepared in skin test diluent and applied intracutaneously to the volar side of the forearms of the patients or appropriate control subjects.^{15,16} Injection sites were at least 5 cm apart from each other. A volume resulting in a wheal about 5 mm in diameter (approximately 0.02 ml) was injected intracutaneously on each site. The reactions were read exactly 15 minutes after allergen challenge. The area of the wheal was outlined with a pen and transferred to the recording sheet with cellophane tape. The wheal area was estimated according to the formula

$$\left(\frac{d_1 + d_2}{2} \right)^2$$

where d_1 and d_2 are the two largest diameters perpendicular to each other. Wheal areas were then plotted against concentration on a log-log scale, and linear regressions were calculated for each allergen in every patient.²² The concentration resulting in a wheal area of 100 mm² (ICC₁₀₀) was calculated for each preparation in all patients. The two allergens to be compared were always tested simultaneously in each patient.

Histamine and the albumin diluent were used as positive and negative controls, respectively. In intracutaneous skin testing with Hymenoptera venoms a test

TABLE I. Comparison of BV and PLA_n in quantitative skin testing

Patient No.	No. of allergen doses	Linear regression		Difference in slope	<i>r</i>	ICC ₁₀₀
		Intercept	Slope			
1						
BV	4	2.98	0.183		0.99†	−5.33
PLA _n	5	2.85	0.122	−0.061	0.96†	−6.95
2						
BV	6	2.53	0.127		0.99‡	−4.15
PLA _n	5	2.61	0.125	−0.002	0.97†	−4.87
3						
BV	6	2.74	0.149		0.99‡	−4.99
PLA _n	5	2.61	0.128	−0.021	0.99*	−4.95
4						
BV	5	2.81	0.169		0.99†	−4.78
PLA _n	5	2.88	0.133	−0.036*	0.99‡	−6.59
5						
BV	4	3.46	0.284		0.96*	−5.14
PLA _n	5	2.93	0.166	−0.118*	0.99†	−5.59
6						
BV	5	2.92	0.201		0.96†	−4.55
PLA _n	5	2.81	0.140	−0.061	0.99†	−5.78
7						
BV	4	2.81	0.234		0.99†	−3.44
PLA _n	5	2.61	0.132	−0.102‡	0.99‡	−4.61
8						
BV	4	2.94	0.219		0.99†	−4.29
PLA _n	5	2.88	0.152	−0.067*	0.99†	−5.76
9						
BV	4	2.79	0.184		0.96*	−4.3
PLA _n	5	2.66	0.132	−0.052	0.98†	−5.01
Mean						
BV	4.7	2.79	0.194		0.99‡	−4.55
PLA _n	5	2.77	0.136	−0.058*	0.99‡	−5.57

**p* < 0.05.

†*p* < 0.01.

‡*p* < 0.001.

result is regarded as positive when a wheal of at least 5 mm in diameter (25 mm² area) with erythema results.¹⁶

Statistical analysis

For linear regression analysis of log wheal area on log allergen concentration we selected only test series in which at least three (generally four to six) wheals of 25 mm² or larger could be measured. The correlation coefficient and the global *F* test were calculated for each test series.²³ To reduce errors resulting from technical inaccuracy during skin testing, only regressions with an *r* greater than 0.90 and a *p* value of less than 0.05 in the global *F* test were further investigated. On the basis of all the described conditions, seven of 16 patients (five with < three wheals) had to be excluded for the comparison of BV with PLA_n, three of seven (three with < three wheals) for PLA_n-PLA_d comparison, and three of

nine (one with < three wheals) for PLA_n-PLA_r comparison.

To compare the relative potency of the various allergen preparations, we proceeded in two steps. First, we compared the two slopes within each patient by calculating their difference and testing it against zero with an *F* test. This was achieved with a regression model with an interaction term of log concentration with allergen. The difference in slopes and the results of this test are displayed in Tables I through III. Next, the slope differences were treated as single observations and it was tested whether their mean differed significantly from zero by a one-sample, two-sided *t* test. If the null hypothesis was confirmed, indicating that the slopes of the regressions were not significantly different, the relative potency was calculated on the basis of a common slope.²⁴ A significant difference in relative potency was

TABLE II. Comparison of PLA_n and PLA_d in quantitative skin testing

Patient No.	No. of allergen doses	Linear regression		Difference in slope	<i>r</i>	ICC ₁₀₀
		Intercept	Slope			
10						
PLA _n	5	2.48	0.158		0.99*	-3.03
PLA _d	5	2.50	0.167	0.009	0.99†	-3.2
11						
PLA _n	5	3.13	0.236		0.96*	-4.79
PLA _d	5	2.66	0.175	-0.061	0.99†	-3.76
12						
PLA _n	4	2.46	0.092		1.0‡	-4.96
PLA _d	5	2.28	0.069	-0.023*	0.99‡	-4.01
13						
PLA _n	4	2.59	0.157		0.99*	-3.73
PLA _d	4	2.80	0.212	0.055	0.98*	-3.79
Mean						
PLA _n	4.5	2.59	0.160		0.99‡	-3.96
PLA _d	4.75	2.48	0.142	-0.018	0.98†	-3.63

Relative potency: PLA_n, 100 (95% confidence limits, 58 to 243); PLA_d, 65.1 (95% confidence limits, 18 to 301).

**p* < 0.05.

†*p* < 0.01.

‡*p* < 0.001.

assumed when the value of the test preparation was not within the 95% confidence intervals found for PLA_n. If the null hypothesis was rejected, indicating nonparallel lines, ICC₁₀₀ was used for an approximate estimation of relative potencies.

To judge the reproducibility of the skin test method, nine patients with BV allergy were tested simultaneously in duplicate with five serial 10-fold dilutions of PLA_n. The mean within-patient variation coefficient for the wheal area was 7.5%; after exclusion of two patients in whom the regression did not fulfill the criterion of an *r* of 0.9 or greater, it was 5.9%. A significant difference in slope was observed in only one patient, who had to be excluded because of an *r* value less than 0.9. The 2 SD limits of the geometric mean relative potency were 54 to 257; the 95% confidence interval was 58 to 243.²⁴

RESULTS

Comparison of type I skin test reactivity to BV and PLA_n

In a screening experiment 58 patients with a history of allergic reactions to bee stings and a positive skin test reaction to BV at 10⁻⁴ gm/L (0.1 µg/ml) were skin tested with PLA_n. All also had a positive test reaction to PLA_n at 10⁻⁴ gm/L. In contrast, 22 control subjects with a negative BV skin test reaction at 10⁻³ gm/L also had a negative skin test reaction to PLA_n at this concentration.

Nine subjects allergic to BV were studied in more detail by quantitative skin testing. Their results are summarized in Table I and Fig. 1.

Comparison of the mean slopes of the linear regressions for BV versus PLA_n by *t* test shows a significant difference (*p* < 0.01), indicating nonparallel lines. The slope for PLA_n was consistently smaller than that for BV. According to the mean ICC₁₀₀, PLA_n was 10.5 times more potent than BV. PLA_n was more potent than BV in eight of the nine patients and of similar relative potency in one (patient 3; Table I).

Comparison of type I skin test reactivity to PLA_n and PLA_d

Four subjects with BV allergy were studied. Their results are summarized in Table II and Fig. 2. It can easily be seen that PLA_n and PLA_d were of similar relative potency in all four patients. The relative potency of PLA_d was calculated to be 65.1% (95% confidence limits, 18 to 301) of that of PLA_n. Statistically, no difference was found between the two regressions regarding either slope or relative potency (*p* > 0.05). However, because of the small sample, these results must be interpreted cautiously.

Comparison of type I skin test reactivity to PLA_n, PLA_r, and PLA_{nr}

Six persons allergic to BV and six control subjects were studied. Table III and Fig. 3 summarize the results of the patients. PLA_{nr} induced only

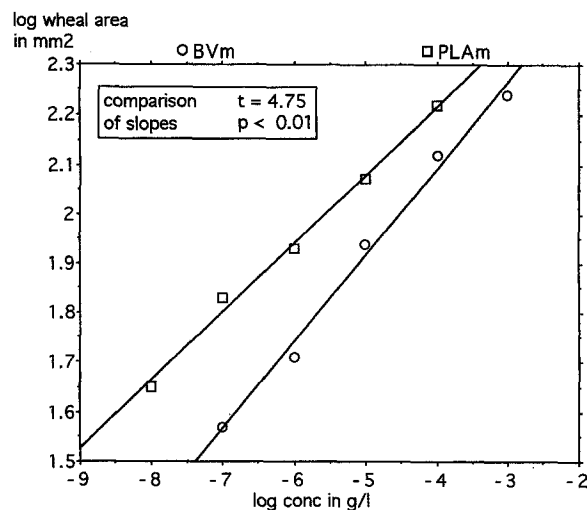


FIG. 1. Comparison of BV and PLA_n in quantitative intracutaneous skin testing. Mean values of nine simultaneously tested patients are given.

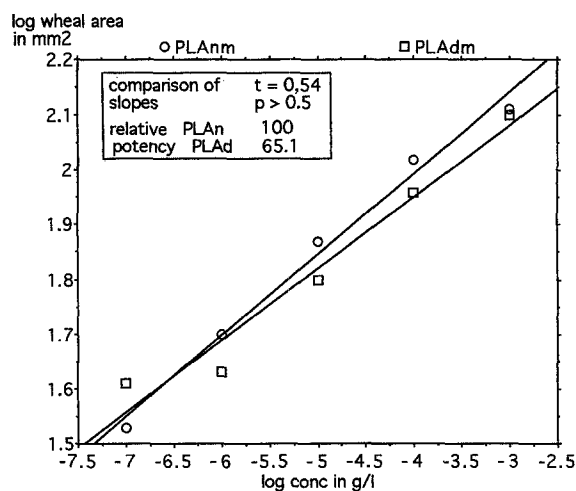


FIG. 2. Comparison of native (PLA_n) and PLA_d in quantitative intracutaneous skin testing. Mean values of four simultaneously tested patients are given.

TABLE III. Comparison of PLA_n and PLA_r in quantitative skin testing

Patient No.	No. of allergen doses	Linear regression		Difference in slope	<i>r</i>	ICC ₁₀₀
		Intercept	Slope			
11						
PLA_n	4	2.45	0.150		1.0‡	-3.0
PLA_r	4	2.31	0.135	-0.015	0.98*	-2.33
13						
PLA_n	3	2.75	0.188		1.0†	-4.0
PLA_r	4	2.15	0.091	-0.097*	0.97*	-1.69
14						
PLA_n	5	2.89	0.156		0.99†	-5.68
PLA_r	5	2.50	0.105	-0.051*	0.98†	-4.77
15						
PLA_n	5	2.98	0.160		0.99‡	-6.12
PLA_r	5	3.04	0.165	0.005	0.97†	-6.25
16						
PLA_n	3	2.87	0.222		0.99*	-3.92
PLA_r	4	2.64	0.152	-0.070	0.99†	-4.24
17						
PLA_n	4	3.12	0.210		0.98*	-5.34
PLA_r	4	2.79	0.143	-0.067	0.99*	-5.49
Mean						
PLA_n	4	2.80	0.181		0.99†	-4.79
PLA_r	4.3	2.53	0.131	-0.050*	0.99†	-4.45

**p* < 0.05.

†*p* < 0.01.

‡*p* < 0.001.

minimal skin test reactivity in two of the six patients at 10^{-2} gm/L and 10^{-1} gm/L, respectively, and no activity at up to 10^{-1} gm/L was found in the remaining four patients (data not shown). In the

six control subjects no reactivity was observed to PLA_n or PLA_r up to 10^{-3} gm/L. Comparison of the mean slopes of the linear regressions for PLA_n versus PLA_r by *t* test shows a significant difference

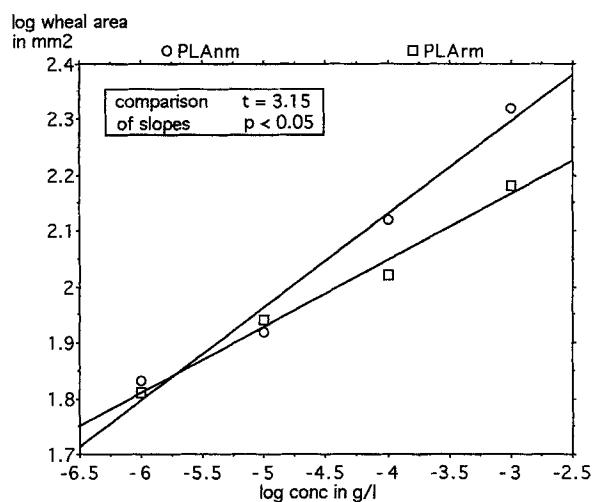


FIG. 3. Comparison of PLA_n and PLA_r in quantitative intracutaneous skin testing. Mean values of six simultaneously tested patients are given.

($p < 0.05$) indicating nonparallel lines: the slope for PLA_r was usually smaller than for PLA_n. According to ICC₁₀₀ values, the relative potencies of the two preparations were similar, with PLA_n being about twice as potent as PLA_r.

DISCUSSION

PLA is the major allergen of BV.¹⁰ Its three-dimensional structure has been resolved by x-ray crystallography,²⁵ and detailed knowledge about its enzymatic activity is available.¹⁹ The gene encoding PLA has been cloned¹² and PLA_r produced.¹³ Although recombinant DNA technology offers great prospects for diagnosis and treatment of allergic disease,⁸ several problems may arise:

1. Allergen extracts are usually mixtures of several major and minor allergenic components. In BV, besides the major allergen PLA, the components hyaluronidase, acid phosphatase, melittin, and allergen C have been shown to induce IgE antibody production in some patients.^{10, 26-28} A single major allergen may therefore be inadequate for diagnostic and therapeutic purposes.
2. Many allergens such as PLA are glycoproteins. About 90% of PLA_n isolated from BV is glycosylated on a single site. The carbohydrate moiety, however, is not expressed in recombinant allergens produced in a prokaryotic system.
3. Allergenic B-cell epitopes are thought to be conformational,²⁹ that is, dependent on the correct three-dimensional structure of the protein molecule. Although the prokaryotic expression system used provides the amino acid se-

quence of the allergen reliably, an in vitro procedure has to be developed to refold the recombinant allergen molecule to its correct three-dimensional structure.¹³

In previous studies^{14, 30} we observed similar activities of PLA_n and PLA_r in in vitro tests such as antibody-binding assays with human IgE and IgG antibodies and in histamine release from peripheral blood leukocytes obtained from patients allergic to BV.

In this article we have used an in vivo method, intracutaneous skin testing, to address the three problems mentioned. In a first series of experiments PLA_n was compared with whole BV. We actually found that none of 58 patients allergic to BV had negative responses to skin testing with PLA_n, thus confirming PLA as the major allergen of BV.^{10, 27, 28} Because none of 22 control subjects with negative BV skin test results reacted to PLA_n, the specificity of PLA_n seems to be at least equal to that of whole BV. The detailed analysis in nine patients showed that the mean relative potency based on ICC₁₀₀ is 10.5 times higher in PLA_n than in BV. This can be expected because PLA constitutes 10% to 15% of the protein content of BV.⁹ It also fits well with the results of our in vitro estimation of the PLA content of the BV stock solution by ELISA inhibition, which was found to be 10% of whole BV protein. However, considerable individual differences were observed, and the slope for PLA_n was significantly smaller than that for BV. Both observations may be explained by the varying contribution of other allergens besides PLA. Although diagnostic skin testing with PLA_n alone could thus replace testing with whole BV without significant loss, immunotherapy with the major allergen alone might still not induce full protection in all patients.

Sugar residues of PLA have been reported to be important for IgE-binding and hence for the allergenicity of this protein.³¹ The recombinant allergen used was not glycosylated. In a second set of experiments we therefore compared PLA_n with PLA_d. In the four persons allergic to BV tested we found similar slopes and an almost identical relative potency of the two preparations. This suggests that the carbohydrate moiety is of little relevance for IgE-binding in these patients. However, it is conceivable that glycosylation is relevant for the induction and maintenance of PLA sensitization.

Comparison of PLA_n and PLA_r showed similar activity in skin testing of these two preparations. PLA_{nr}, on the other hand, had only minimal

activity in two of six patients and none in the other four. Correct refolding is thus a prerequisite not only for the enzymatic activity of PLA but also for the *in vivo* activity of this molecule as an allergen. These results indicate that PLA-specific IgE antibodies recognize mainly conformational epitopes. The slopes of linear regressions were significantly smaller for PLA_r than for PLA_n. This observation could be explained by minor conformational differences between PLA_n and PLA_r, even though no difference between the two molecules was found when the enzymatic function,^{13, 19} the *in vitro* binding to phospholipid bilayers, or the circular dichroism spectra (Suter M, Gelb HM, unpublished observations) were compared. Contamination of PLA_n with minor allergens could therefore be a more likely explanation for the steeper slope of PLA_n, analogous to the observation that whole BV consistently resulted in a steeper slope than PLA_n.

Overall, our results from skin testing are similar to those found in various *in vitro* assays. Thus PLA_n and PLA_r inhibited binding of both IgE and IgG4 antibodies to PLA_n in a similar way, whereas PLA_{nr} did not inhibit at all, even at high concentrations.¹⁴ Likewise, comparable histamine release from basophils of subjects allergic to BV was observed with PLA_n and PLA_r, but no release at all was found with PLA_{nr}.³⁰

Two conclusions can be drawn from our experiments:

1. It is possible to produce recombinant allergens with an *in vivo* activity similar to that of purified native allergens. This may be especially valuable in situations where the isolation of natural allergenic fractions is notoriously difficult, such as in fungi.³² In these cases potent major allergens present in only small amounts in the natural extract can be produced in large amounts as recombinant allergens. This possibility may become crucial for the improvement of diagnosis of various allergies.⁷ The availability of biologically active recombinant allergens together with genetic engineering also allows definition and localization of discontinuous antibody-binding epitopes on the molecule and identification of epitopes responsible for cross-reactivities between allergens from different biologic sources.³³
2. PLA_{nr} is enzymatically inactive and thus non-toxic to cells. It is also unable to elicit allergic reactions *in vivo*. This may be advantageous for the study of T cell-mediated immune responses, which are thought to be induced by nonconfor-

mational, continuous amino acid sequences and which may be disturbed by the cytotoxic action of the native molecule.³⁴ The lack of cytotoxicity and the inability to elicit immediate-type allergic *in vivo* reactions could also prove valuable for immunotherapy for allergic diseases. Further *in vitro* and *in vivo* studies, however, are needed to show that unfolded allergens or even T-cell epitope-containing peptide fragments thereof³⁵ are actually capable of inducing anergy or modifying the immune response from allergy to immune protection.

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