

Increased specificity of diagnostic tests with recombinant major bee venom allergen phospholipase A2

U. MÜLLER, M. FRICKER, D. WYMAN*, K. BLASER* and R. CRAMERI*

Medical Division, Zieglerspital Bern and *Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

Summary

Background In diagnosis of type I allergy recombinant allergens have potential advantages over conventional allergenic extracts, both regarding specificity and reproducibility.

Objectives We therefore decided to study honey bee venom (BV) and its major allergen phospholipase A2 (PLA) in native and recombinant form for diagnosis of bee sting allergy.

Method We investigated 85 patients with a history of a recent systemic allergic bee sting reaction and positive intracutaneous skin test to BV, and 21 controls with no history of allergic bee sting reaction and negative skin test to BV. Intracutaneous skin tests and determination of specific IgE by ImmunoCAP^R to BV, native PLA (nPLA) and recombinant PLA (rPLA) were done in all patients and controls.

Results In skin testing 84 (99%) of the 85 patients reacted to nPLA and 81 (95%) to rPLA, while none of the 21 controls was positive with nPLA or rPLA. Specific serum IgE to BV could be detected in 82 of the patients (96%), to nPLA in 73 (86%) and to rPLA in 66 (78%). Four (19%) of the controls had a positive CAP to BV, one (4.8%) to nPLA and none to rPLA. Analysis of discordant results in CAP showed, that most patients with specific IgE to BV, but not to nPLA and rPLA, had positive skin tests to both PLA preparations and low levels of BV specific IgE. Patients with specific IgE to nPLA but not to rPLA were usually sensitized to minor allergens of BV which contaminated the commercial nPLA.

Conclusions PLA is the major allergen in BV. While diagnostic tests with BV are more sensitive, the specificity of tests with PLA, especially rPLA is clearly increased as compared with BV.

Keywords: bee venom allergy, skin tests, specific IgE, phospholipase A2, recombinant allergens

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Introduction

Systemic allergic reactions to honey bee stings occur in about one percentage of the population in Western Europe [1]. Most often these are IgE-mediated and rarely they can be fatal. The major allergen in honey bee venom is phospholipase A2 (PLA) [2,3]. PLA has been cloned [4] and expressed in a prokaryotic system [5]. The recombinant PLA (rPLA) produced in *E. coli* and refolded *in vitro* was shown to be enzymatically active [6] and to possess full *in*

vitro and *in vivo* antibody binding properties [3,7,8] similar to those of the native PLA (nPLA). The potential advantage of recombinant allergens consists in their illimited availability in identical form, allowing optimal standardization, and the absence of contamination by traces of other allergens which might confound the true relevance of an individual allergen for the respective allergic disease [9].

In this study we evaluated the diagnostic reliability of rPLA in comparison with nPLA and whole BV. In a previous study [3] we have observed minor differences between nPLA and rPLA, notably a significant difference in the slope of the dose–response curve in skin testing.

Correspondence: Dr U. R. Müller, Medical Division, Zieglerspital, CH-3007 Bern, Switzerland.

The question arose, whether this difference was due to structural defects in the recombinant preparation such as incorrect folding or lack of glycosylation, or rather to contamination of the native, purified PLA with minor BV allergens.

Here we analyse these questions on the basis of intracutaneous skin testing and determination of specific IgE serum antibodies to BV, nPLA and rPLA in a large number of BV allergic patients and a control group of non-allergic individuals. We show that PLA is the major allergen of BV and that diagnostic tests, especially with rPLA, have increased specificity as compared with whole BV.

Materials and methods

Patients and controls

We investigated 85 patients with a history of recent systemic allergic bee sting reaction and a positive i.c. skin test to BV at 10^{-3} g/L ($1 \mu\text{g/mL}$). Twenty-one individuals with no history of systemic sting reactions and a negative i.c. skin test at 10^{-3} g/L served as controls.

Allergens

BV for skin testing (Pharmalgen) was provided by ALK, Horsholm, Denmark; for PAGE and Western blot by Sigma, St. Louis, Missouri, USA. nPLA for skin testing was obtained from Boehringer, Mannheim, Germany. rPLA was prepared as described previously [5]. Purified hyaluronidase was kindly provided by Dr D. Kemeny, London, UK. For skin testing, allergens were dissolved in commercially available skin test diluent (Pharmalgen, ALK, Horsholm, Denmark). The experimental preparations nPLA and rPLA were sterile filtered before use. For estimation of specific serum IgE to BV and nPLA commercial CAPs, kindly provided by Pharmacia-Upjohn, Switzerland, were used. We thank Dr Hans Grönlund, Pharmacia-Upjohn, Uppsala, Sweden, for the preparation of experimental CAPs coupled with rPLA.

Skin tests

All patients and controls were injected with 0.02 mL of the concentrations 10^{-8} , 10^{-6} , 10^{-4} and 10^{-3} g/L of BV, nPLA and rPLA i.c. on the volar side of the forearm as described previously [8]. A weal of ≥ 5 mm in diameter with erythema at a concentration of $\leq 10^{-3}$ g/L was considered as a positive reaction.

The lowest concentration resulting in such a reaction, the endpoint concentration (EPC), was used as a semi-quantitative measure of the skin sensitivity to the respective allergen.

Determination of specific serum IgE

Specific serum IgE antibodies to BV, nPLA and rPLA were measured by the ImmunoCAP^R system (Pharmacia-Upjohn, Uppsala, Sweden). All tests were done in duplicate. The results are given in kU/L or CAP classes from 0 to 6. A value of ≥ 0.35 kU/L (CAP class 1–6) was regarded as a positive result. RAST-inhibition assays were performed as described previously [10].

Polyacrylamide gel electrophoresis (PAGE) and Western blot

BV at a concentration of 10 mg/mL ($80 \mu\text{g/cm}$) was separated in a 17.5% polyacrylamide gel at 200 V for 45 min using a BioRad^R mini gel electrophoresis system (BioRad^R, CA, USA). The separated bands were blotted onto nitrocellulose in transfer buffer for 1 h at 100 V/250 mA and then blocked by incubation with TBS/casein. Thereafter, 4 mm strips were cut and incubated overnight in a 1:5 solution of patient serum in TBS. Strips were then washed and incubated for 18 h in a 1:1000 dilution of peroxidase coupled anti-human IgE MoAb BSW 17 (kindly provided by Dr Beda Stadler, Bern, Switzerland). After extensive washing with TBS, IgE-binding bands were visualized by incubation in chloronaphthol substrate solution for 1 h.

Statistics

For calculation of correlation (Pearson's r) of skin sensitivity to the three allergens, the negative log of the EPC was used, for correlation between specific serum IgE log transformed CAP values in kU/L. Mean values obtained with the three allergens were compared by paired t -test. Again untransformed data were used for EPC, log transformed values for specific IgE.

Results

Comparison of skin tests with BV, nPLA and rPLA

Of the 85 BV allergic patients with a history of a systemic bee sting reaction and positive skin test to BV, 84 (99%) had a positive skin test also with nPLA, 81 (95%) with rPLA. The EPC with both PLA preparations was usually at least 1 log lower than with BV. None of the history and BV skin-test negative controls reacted with nPLA or rPLA. Correlations between the EPCs obtained with the three allergens were significant for all comparisons (BV/nPLA, $r = 0.427$; BV/rPLA, $r = 0.474$; nPLA/rPLA, $r = 0.82$). Skin sensitivity as indicated by EPC was significantly higher to nPLA and rPLA than to BV as shown in Table 1. Thus, the sensitivity and specificity of skin tests to all three preparations was very high.

Table 1. Comparison of mean skin sensitivity and specific serum IgE to BV, nPLA and rPLA

	BV	nPLA	rPLA	BV/nPLA	BV/rPLA	nPLA/rPLA
Skin sensitivity						
MV* (–log EPC)	5.51	7.18	6.65			
SD	1.6	1.58	1.99			
Range	3–8	2–8	2–8			
Paired <i>t</i> -test				–9.06	–5.801	4.49
<i>P</i>				<0.0001	<0.0001	<0.0001
Specific IgE						
MV** (kU/L)	11.69	4.85	4.42			
SD	3.72	3.16	3.49			
Range	0.3–100	0.3–81	0.3–100			
Paired <i>t</i> -test				13.22	10.29	1.88
<i>P</i>				<0.0001	<0.0001	NS

* arithmetic mean value; ** geometric mean value.

Table 2. Results of i.c. skin tests (ST) and estimation of specific serum IgE in 85 bee venom allergic patients and 21 control individuals

Population	Test	<i>n</i>	Number positive with (%)		
			BV	nPLA	rPLA
Patients	ST	85	85	84 (99)	81 (95)
	CAP	85	82	73 (86)	66 (78)
Controls	ST	21	0	0	0
	CAP	21	4 (19)	1 (4.8)	0

Comparison of specific IgE to BV, nPLA and rPLA

Specific IgE to BV was detected in 82 (96%) of the 85 BV allergic patients, to nPLA in 73 (86%) and to rPLA in 66 (78%). Four of the negative controls had low levels of specific IgE to BV (0.38–2.2 kU/L, CAP classes 1 and 2). One control showed low levels of nPLA specific IgE (0.58 kU/L, CAP class 1) and none to rPLA (Table 2). Correlation between specific IgE to the three allergen preparations are shown in Fig. 1. It is close between BV and nPLA ($r = 0.88$), BV and rPLA ($r = 0.77$) and nPLA and rPLA ($r = 0.93$). Geometric mean values of specific IgE were significantly higher to BV than to nPLA and rPLA (Table 1). As compared with skin tests with BV as the gold standard, sensitivity was somewhat lower for specific serum IgE, especially to rPLA and nPLA, while the specificity was optimal for rPLA but reduced for nPLA and especially whole BV.

Analysis of patients with discordant results in CAP

Patients with discordant CAP results were further analysed according to their skin test results and IgE-binding to individual BV allergens in Western blot. Figure 2, shows IgE immunoprints of patients with high IgE levels to all three allergen preparations and prominent bands at 16–20 kDa (PLA), 35 kDa, 43 kDa (hyaluronidase) and about 50 kDa (acid phosphatase).

Eight out of nine patients with a positive CAP to BV and a negative CAP to nPLA and rPLA, had low serum levels of BV-specific IgE between 0.35 and 1.9 kU/L (CAP class 1 and 2). All had a positive skin test to nPLA at an equal or lower EPC than to BV, but two had a negative skin test to rPLA. In Western blot, one of these two patients, with a CAP of class 3 (6 kU/L) to BV, showed IgE-binding exclusively to the 35 kDa band (not shown), whereas this method was not sensitive enough to detect specific IgE in the remaining eight patients.

Six out of seven patients with a positive CAP to BV and nPLA but a negative CAP to rPLA had high levels of BV specific IgE of 5.8–83 kU/L (CAP class 3–5), one of 2.9 (CAP class 2). Six of the seven showed a positive skin reaction to rPLA. In Western blot (Fig. 2, left) IgE binding to the 35 kDa protein was observed in five patients, to hyaluronidase in two and to PLA in one. These findings indicate a higher sensitivity of skin tests than of CAP and of BV CAP than of CAP to the two PLA preparations. In the RAST-inhibition assay high concentrations of nPLA inhibited binding of specific IgE to purified hyaluronidase completely, while no significant inhibition was observed with the same concentrations of rPLA (data not shown). This demonstrates, that nPLA – in contrast to rPLA – contains traces of contaminating hyaluronidase.

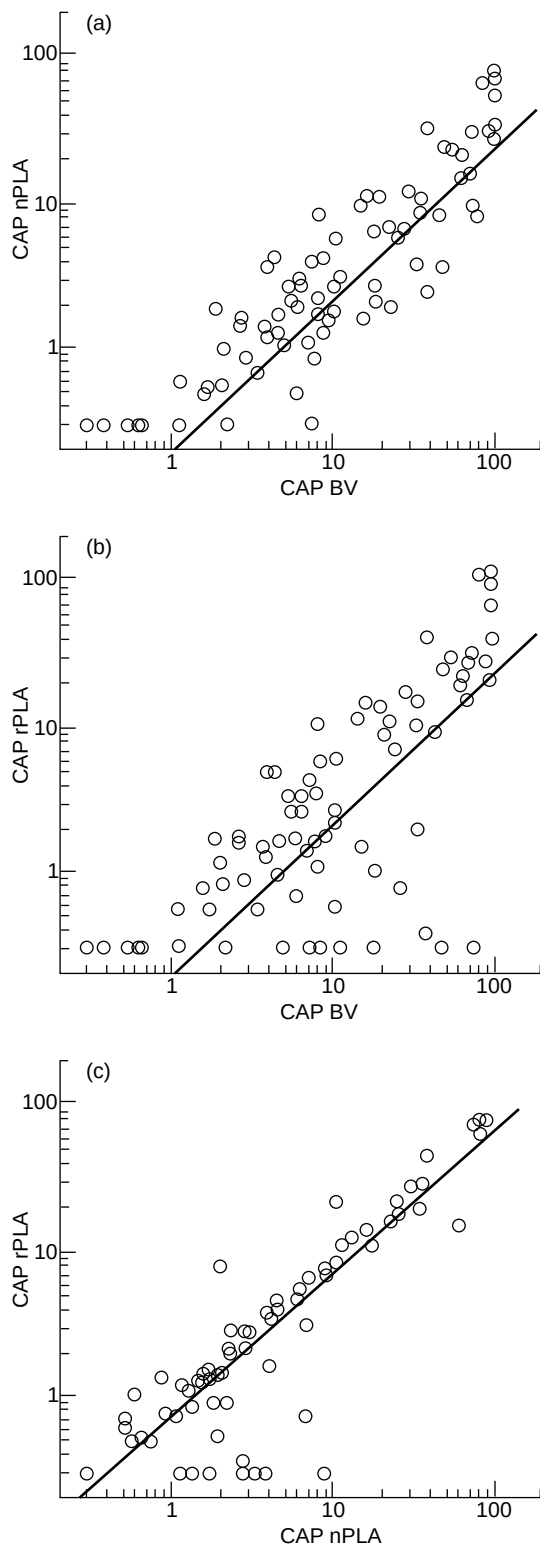


Fig. 1. Correlation of specific serum IgE values in 85 BV allergic patients obtained by CAP (a) between BV and nPLA ($r = 0.884$) (b) between BV and rPLA ($r = 0.771$) and (c) between nPLA and rPLA ($r = 0.932$).

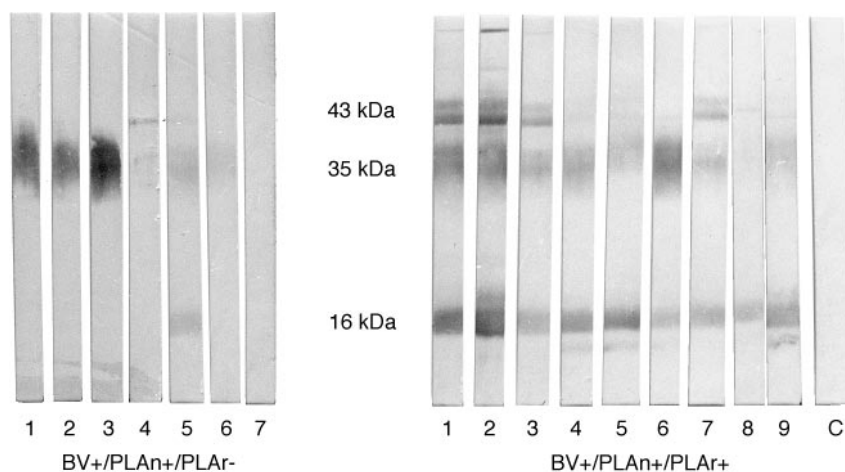
Discussion

Intracutaneous skin tests with BV and determination of BV-specific serum IgE antibodies are the acknowledged diagnostic parameters for confirming IgE-mediated allergy to honey bee stings [11,12]. These tests distinguish well between individuals with and without a history of systemic allergic reactions to honey bee stings. Skin tests are generally more sensitive than determination of BV-specific IgE. Only a small minority, of less than 5% of history positive individuals, is negative in both tests if investigated within a year after a systemic sting reaction. On the other hand, up to 20% of history negative individuals may react positive to either or both tests [13]. Prospectively, test positive individuals will develop a systemic reaction more frequently than test negative when restung, but only part of the test positive patients will react again. Moreover, some test negative individuals may develop a systemic reaction as well [13–15]. The possible reasons for this seeming inaccuracy of diagnostic tests with Hymenoptera venoms are manifold: change of sensitivity – either increase or decrease – since last sting [11]; large variability of venom amount applied with one sting, especially in vespids [16,17]; lack of sensitivity and/or specificity of tests. The availability of recombinant venom allergens promises especially an increase in specificity of diagnostic tests.

In this study patients and control individuals were selected on the basis of a history of systemic allergic bee sting reaction and positive skin tests with BV. Sensitivity and specificity of skin tests with BV can therefore not be judged. It is obvious from the skin test results that PLA must be the major allergen of BV, since almost all patients were also positive to native as well as to recombinant PLA (99 and 95%, respectively). Mean skin sensitivity as indicated by EPC was more than 10 times higher to PLA than to BV. This is not surprising, because concentrations of skin test solutions are based on protein content. Since PLA accounts only for about 10% of the protein of BV, the PLA content of the BV skin test solution is about 10 times lower than that of the solutions of nPLA or rPLA. The higher skin sensitivity to PLA than to BV is therefore additional evidence that PLA is the major allergen of BV and confirms previous observations [2,18,19].

Determination of specific serum IgE resulted in more prominent differences between the three allergens. While sensitivity was near to 100% with BV, it was definitely lower with nPLA and rPLA (86 and 78%, respectively). Conversely, specificity was optimal with rPLA, while almost 20% of history and skin-test negative controls were positive at low levels with BV, which did not allow further analysis with the less sensitive Western blot technique. The clinical relevance of such positive diagnostic tests in history and skin-

Fig. 2. Right: Western blots with sera from nine patients with high levels of specific IgE to BV, nPLA and rPLA are shown. IgE binding to allergens at 16–20 kDa (PLA), about 35 kDa, 43 kDa (hyaluronidase), and sometimes to high molecular weight components are present. C indicates a negative control. Left: Western blots of sera from seven patients with positive CAP to BV and nPLA, but negative CAP to rPLA. Only one (patient 5) has IgE binding to PLA, five to either the 35 kDa allergen and/or to hyaluronidase. In one (patient 7) no IgE binding can be detected.



test negative individuals has not been studied prospectively so far [1,13,20].

The correlation of the IgE values obtained with the three allergens was closest between the two PLA-preparations, and highly significant also between BV and the two PLAs. In contrast to skin sensitivity, mean values of specific IgE were significantly higher to BV than to the PLA preparations, but not significantly different between these two. This apparent discrepancy is most likely due to methodological differences. As mentioned above, the higher skin sensitivity to PLA as compared with BV resulted from the about ten times higher amount of PLA given with PLA- than with BV-skin test solutions. The PLA-CAPs on the other hand were loaded with less protein as compared to the BV-CAPs. As an alternative explanation one could speculate that PLA specific IgE Abs on skin mast cells, representing the majority of BV-specific IgE, are more likely to be crosslinked than IgE Abs to any minor allergen, while minor and major allergens are equally accessible for specific IgE Abs on CAPs. Finally, coupling of PLA to a solid support such as the CAP may make inaccessible part of its IgE-binding epitopes. It was demonstrated that lysine residues in PLA, which are chemically linked to the solid support, are substantially involved in IgE binding [6].

Patients with positive CAP to BV and negative CAP to PLA showed always positive skin tests to nPLA and most often also to rPLA. This indicates that most of them were actually sensitized to PLA, but that the CAP for PLA was less sensitive than for BV. It also reflects the lower sensitivity of the CAP as compared with skin tests. Eight of nine patients with positive CAP to BV but negative CAP to PLA had low levels of BV specific IgE making further analysis by the less sensitive Western blot impossible. In one patient a monovalent sensitization to a 35 kDa allergen could be demonstrated with this technique, indicating that this patient was not sensitized to PLA. The nature of this 35 kDa

allergen, which could be detected in more than 50% of the patients with IgE to whole BV, is not yet clear.

In contrast, six of the seven patients with positive CAP to BV and nPLA but negative CAP to rPLA had high levels of BV specific IgE. All six reacted to either the 35 kDa allergen and/or to hyaluronidase, indicating that the positive results with nPLA were due to contamination of this preparation with minor allergens.

This was further confirmed by RAST-inhibition, where nPLA completely inhibited binding of IgE to hyaluronidase [21]. Only in one patient IgE bound also to the PLA band, suggesting that he may be sensitized to a special epitope, to which the carbohydrate [22,23] — which is not expressed in rPLA — contributed, e.g. by influencing the three-dimensional structure of PLA in a way to create an additional epitope.

In conclusion, even when using highly purified recombinant allergens, we confirmed PLA as the major allergen in BV. We further showed that working with purified, in particular recombinant allergens definitely increases specificity of diagnostic tests, although their sensitivity may suffer. This however, may be solved by improving the coupling procedure without involving lysine residues. Optimal sensitivity and specificity will most likely be obtained with mixtures of a panel of recombinant, major and minor allergens. Finally, we demonstrated, that even highly purified allergenic components, in contrast to recombinant allergens, contain trace amounts of other allergens. The actual clinical relevance of an individual allergen out of an allergen mixture can therefore only be evaluated reliably by using recombinant allergens.

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