

Natural and recombinant enzymatically active or inactive bee venom phospholipase A₂ has the same potency to release histamine from basophils in patients with Hymenoptera allergy

Elisabeth Förster, MD,^a Thomas Dudler,^b Michael Gmachl, PhD,^c
Werner Aberer, MD,^a Radvan Urbanek, MD,^a and Mark Suter, DVM^b
Vienna, Austria, Davos-Platz, Switzerland, and Salzburg, Austria

Background: A complementary DNA encoding the major bee venom allergen phospholipase A₂ (PLA) has been characterized recently. Recombinant PLA was produced in *Escherichia coli* and purified to apparent homogeneity. Natural PLA was compared with recombinant PLA in its ability to release histamine from blood basophils.

Methods: A synthetic gene encoding the mature form of PLA was expressed in *E. coli*, and the polypeptide was purified to homogeneity by affinity chromatography and refolded, yielding fully enzymatically active PLA. In addition, we have produced a genetically engineered enzymatically inactive variant by substitution of a single amino acid residue in the catalytic center. A standard histamine release assay was used to compare the potency of natural PLA with correctly folded enzymatically active and inactive recombinant PLA to release histamine from blood basophils of nine patients with bee venom allergy.

Results: Recombinant enzymatically active PLA and purified natural protein were equally effective in releasing histamine from sensitized basophils. By comparing the histamine-releasing capacity of enzymatically active and inactive recombinant allergen, we further demonstrate that catalytic activity is not a requirement for allergenicity in the effector phase. Denaturation of natural PLA or incorrect folding of recombinant protein resulted in a total loss of allergenic potency.

Conclusion: We demonstrate the feasibility of producing native-like recombinant allergens with or without enzymatic activity. We also provide evidence for the requirement of correct three-dimensional structure of PLA to induce histamine release from basophils and thus evidence for its recognition by IgE. (*J ALLERGY CLIN IMMUNOL* 1995;95:1229-35.)

Key words: Histamine release, recombinant allergen, glycosylation, bee venom phospholipase A₂

Diagnosis of type I allergies by procedures such as RAST, histamine release (HR) assay, or skin

From ^athe Pediatric and First Department of Dermatology, University of Vienna; ^bthe Swiss Institute of Allergy and Asthma Research, Davos-Platz; and ^cthe Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg.

Supported by the Swiss National Science Foundation, grant NSF 31-28815.90. Dr. Förster was supported by an Otto Loewi Scholarship of the Austrian Science Research Fund no. K54-MED.

Received for publication June 9, 1993; revised Sept. 7, 1994; accepted for publication Nov. 17, 1994.

Reprint requests: Elisabeth Förster, MD, Allgemeines Krankenhaus, Universitäts-Kinderklinik, Währinger Gürtel 18-20, 1090, Wien, Austria.

Copyright © 1995 by Mosby-Year Book, Inc.
0091-6749/95 \$3.00 + 0 1/1/62171

Abbreviations used

HR:	Histamine release
PLA:	Honeybee venom phospholipase A ₂
nPLA:	Biochemically purified natural PLA
rPLA:	Recombinant PLA
rPLAm:	Recombinant PLA mutated at amino acid position 34 from histidine to glutamine

testing requires standardized protein preparations. In the past, natural allergens purified to various degrees were used for this purpose. However, natural protein preparations are difficult to stan-

dardize, and it has been shown that some commercial extracts lack allergenic components.^{1,2}

Recent improvements in recombinant DNA technology promise the availability of pure allergens in unlimited amounts and of reproducible quality.³ Thus the question arises as to whether this recombinant material could replace the laboriously purified natural allergens for diagnostic applications and possibly for therapy.

Bee venom is a complex mixture of proteins, peptides, and biogenic amines.^{4,5} Some of these molecules have allergenic potential, mainly phospholipase A₂ (PLA), hyaluronidase, and mellitin. PLA is considered to be the major allergen.^{5,6} A complementary DNA encoding PLA has recently been cloned from an expression library of worker bee venom glands.⁷ A synthetic gene encoding the mature protein was designed and expressed in *Escherichia coli*, recombinant protein was purified to homogeneity by affinity chromatography and refolded to obtain fully enzymatically active PLA.⁸ We have previously demonstrated that natural and recombinant PLA have equal antigenicity for binding of monoclonal and polyclonal antibodies.⁸⁻¹⁰

However, before the application of recombinant allergens in vivo, the biologic activity of these proteins must be analyzed in vitro. Therefore we compared the allergenic potency of recombinant and natural bee venom PLA in HR studies with blood from patients with bee venom allergy and appropriate controls. In addition, the role of the enzymatic function in allergenicity was elucidated. Therefore a point-mutated allergen lacking catalytic activity was compared with its wild type counterpart. This mutation also eliminated the potential in vivo toxic effects associated with enzymatic activity.¹²

METHODS

Patients

Patients included in the study ($n = 9$) had reported an allergic reaction after bee sting in the past 2 years and had positive skin prick test results. The RAST class against bee venom of these patients was 2 or higher. Atopic ($n = 4$) persons not sensitive to bee venom served as control subjects. None of the patients or subjects tested was receiving medication, and all gave informed consent before they participated in this study.

Preparation of natural and recombinant PLA

Purified natural PLA was a gift from Prof. Leopold März and Dr. Erika Staudacher (Institut für Chemie, Universität für Bodenkultur, Vienna). Aliquots of the same sample frozen at -20°C were used throughout this study. Each aliquot was used once. Some of the natural

PLA was denatured by reduction and carboxymethylation according to a standard procedure.¹³

Recombinant enzymatically active PLA (rPLA) was prepared as described previously.^{8,9} Briefly, a synthetic gene encoding the mature form of PLA, based on the amino acid sequence deduced from the published cDNA sequence, was designed.⁷ This gene was inserted into a bacterial high-level expression vector¹⁴ to produce a fusion protein with six adjacent histidine residues at the amino terminal end, allowing single-step affinity purification.¹⁵ Expression of this [His]₆-PLA construct in *E. coli* yielded 50 mg of fusion protein per liter of culture after affinity purification. The protein showed an apparent molecular weight of 17 kD^{8,9} and was homogeneous when analyzed on a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel. To test the reproducibility of recombinant allergen production, two different preparations (batches 1 and 2) of rPLA were used in the experiments. For this test, two bacterial cultures producing rPLA were processed individually on two occasions as published,^{8,9} tested enzymatically,^{8,9} or as described in the Results section.

For some experiments enzymatically inactive recombinant PLA (rPLAm) was produced by substitution of a conserved histidine residue in the active center with glutamine.^{8,9,12} rPLAm lacked enzymatic activity but had antigenic properties comparable to those of natural PLA or recombinant, enzymatically active PLA, as analyzed by binding to human monoclonal or polyclonal antibodies.⁸⁻¹⁰

Determination of protein concentrations and analysis of phospholipase activity

Protein concentrations were determined with a commercially available kit (Bio-Rad Laboratories, Richmond, Calif.). Phospholipase activity of the different PLA preparations was determined with a spectrophotometric assay as described previously.^{8,9}

HR assay

Each of the PLA preparations was diluted in an HR reaction buffer, as described previously,¹⁶ to concentrations ranging from 10 $\mu\text{g/ml}$ to 0.1 ng/ml . HR assays were performed with a commercial kit (Immunotech, Marseilles, France) according to the manufacturer's recommendations. Briefly, 100 μl of heparinized whole blood, diluted 1:4 in the reaction buffer, was incubated in triplicate samples with 50 μl of allergen solution in 96-well plates (Costar, Cambridge, Mass.) for 30 minutes at 37°C . To verify mediator releasability from basophils analyzed, release by anti-IgE was included as a positive control with standard procedures.¹⁶ The reaction was stopped by cooling the sample to 4°C , and supernatants were recovered by centrifugation. Total histamine content of cells was determined after freeze-thaw lysis. Spontaneous HR was evaluated by incubating the samples with buffer alone. Histamine in the samples was acylated before the radioimmunoassay was per-

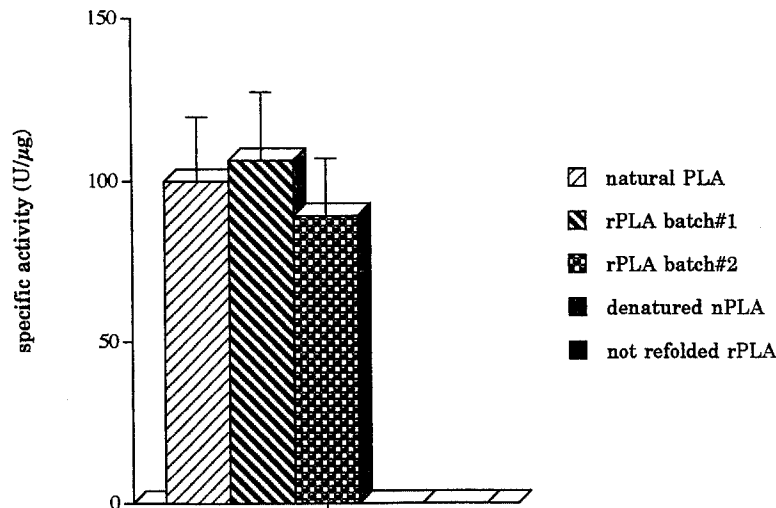


FIG. 1. Catalytic activity of native and recombinant PLA. Identical amounts of PLA were analyzed by enzymatic assay. Natural PLA was arbitrarily set as 100 U specific activity per microgram of protein.

formed according to the procedure recommended by the manufacturer (Immunotech). Incubation of samples with iodine 125-labeled tracer was carried out overnight at 4° C. Free tracer was decanted, and radioactivity was determined.¹⁷ Amount of histamine in the samples was calculated by interpolation of the linear regression plot obtained with a reference sample.

Statistical analysis

The HR data were analyzed with a standard statistical program package. For the determination of the intraassay variations generated by each of the PLA preparations, HR analysis was done in triplicate with a single source of blood; HR was triggered by various amounts of one PLA preparation at a time. The mean HR values were thus calculated from the data points obtained, and relative standard deviations of the mean (percent) were deduced. To test the effectiveness of HR from blood basophils triggered by the various allergen preparations, the triplicate data sets of HR values were averaged first, and then the relevant data points were analyzed by a paired *t* test.

RESULTS

A diagnostic or potential therapeutic application of recombinant allergens requires the availability of recombinant protein preparations with properties comparable to those of the purified natural counterpart. In this study we took advantage of the extensive biochemical knowledge of PLA, which was used as the basis for the comparison between the natural and the recombinant allergen.

The enzymatic activity of the PLA molecule is strictly dependent on the correct three-dimensional structure of PLA,^{8,9} and thus this function

of the protein was tested to verify native-like folding of the recombinant protein (Fig. 1). Natural PLA or two different batches of refolded enzymatically active PLA (rPLA) showed similar enzymatic activity, whereas denatured natural PLA or nonrefolded rPLA did not show any detectable enzymatic activity. rPLA was used as a fusion protein in these studies.^{8,9}

Antigenic studies comparing natural and recombinant enzymatically active PLA molecules by using human monoclonal or polyclonal antibodies revealed no difference between the molecules.⁸⁻¹⁰ This was confirmed with natural PLA and with both batches of rPLA, as well as with rPLAm (data not shown). Denatured or nonrefolded PLA did not bind to the same antibodies (data not shown). These findings indicate that the integrity of the three-dimensional structure is a prerequisite for both the catalytic activity and the antigenic function and suggest a native-like folding of rPLA and rPLAm.

Recombinant and natural PLA show similar histamine-releasing capabilities

Various concentrations of recombinant (batch 1) and natural PLA were compared for their capacity to release histamine from blood basophils of patients with bee venom allergy. A typical example of dose-dependent HR from basophils of one patient with allergy is shown in Fig. 2. Spontaneous HR was below 7% in all samples and was subtracted from the actual values. Natural or refolded rPLA released between 20% and 45% of

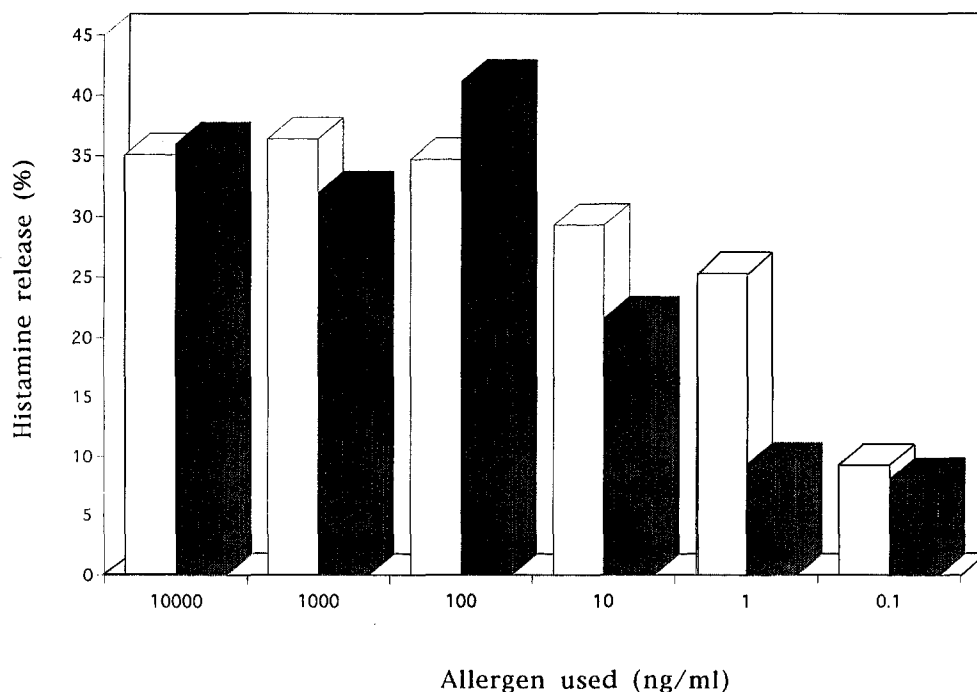


FIG. 2. HR from basophils of a single individual with natural or rPLA as triggering allergen. Concentrations ranging from 10^4 ng/ml to 0.1 ng/ml of natural PLA (*bar 1*) or rPLA (*bar 2*) were used, and the percent specific HR was plotted. The mean of triplicate values is shown.

total histamine at PLA concentrations ranging from 10^4 to 10^2 ng/ml in all patients tested. In general, HR increased gradually from 0.1 ng/ml to 100 ng/ml and reached a maximum at 100 ng/ml to 10 μ g/ml of PLA.

No significant HR in response to natural or rPLA was triggered when basophils from four atopic individuals not allergic to bee venom were used (data not shown).

The intraassay variations of the test were approximately plus or minus 21% for natural PLA or for rPLA. Data from three subjects were included in this analysis. The variations within the test agree with other HR systems.¹⁸

To test the reproducibility of recombinant allergen preparation, two different batches of rPLA were used (Fig. 3). There was no significant difference in HR between the two batches of rPLA. The natural and recombinant PLA preparations were further analyzed statistically to evaluate differences in the capacity to release histamine. All data points available from nine patients with bee venom allergy were used for this analysis. There was no statistically significant difference between the PLA preparations analyzed (data not shown). The data further indicate that PLA used at a concentration of 1000 ng/ml was best for routine use because of

the maximal HR and good reproducibility (Fig. 1 and Fig. 4, *A*).

Enzymatic inactive, correctly folded recombinant PLA and rPLA release similar amounts of histamine from basophils

Cell toxic effects associated with enzymatic activity of PLA have been reported.¹¹ This may be undesirable if PLA were to be used therapeutically. We thus compared rPLA to rPLAm, a genetically engineered mutant lacking enzymatic activity in its capacity to release histamine from basophils obtained from five patients. The dose-dependent release by rPLAm paralleled the release induced by rPLA (Fig. 3). Analysis of the data is presented in Fig. 4, *B*. There was no significant difference between rPLA and rPLAm, indicating that the enzymatic activity was not required for IgE-mediated mediator release and did not enhance HR. We did note that, in general, rPLAm triggered the least amount of histamine when compared with the other PLA molecules. The data further substantiate that a three-dimensional structure similar to rPLA or natural PLA was obtained with rPLAm (Fig. 3 and Fig. 4, *B*). In contrast, nonrefolded rPLA or denatured natural PLA was unable to induce any significant HR (data

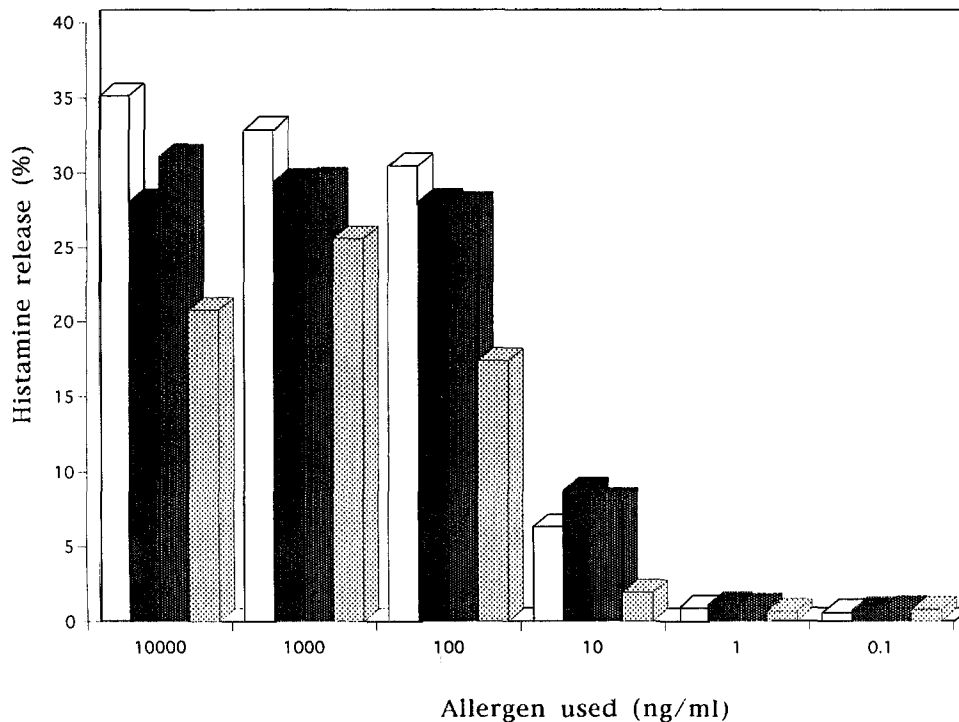


FIG. 3. HR from basophils of a single individual with natural PLA and two batches of rPLA and rPLAm as triggering allergens. The experiment was performed as described in the legend to Fig. 1. Natural PLA (*bar 1*), rPLA (*bars 2 and 3*), rPLAm (*bar 4*).

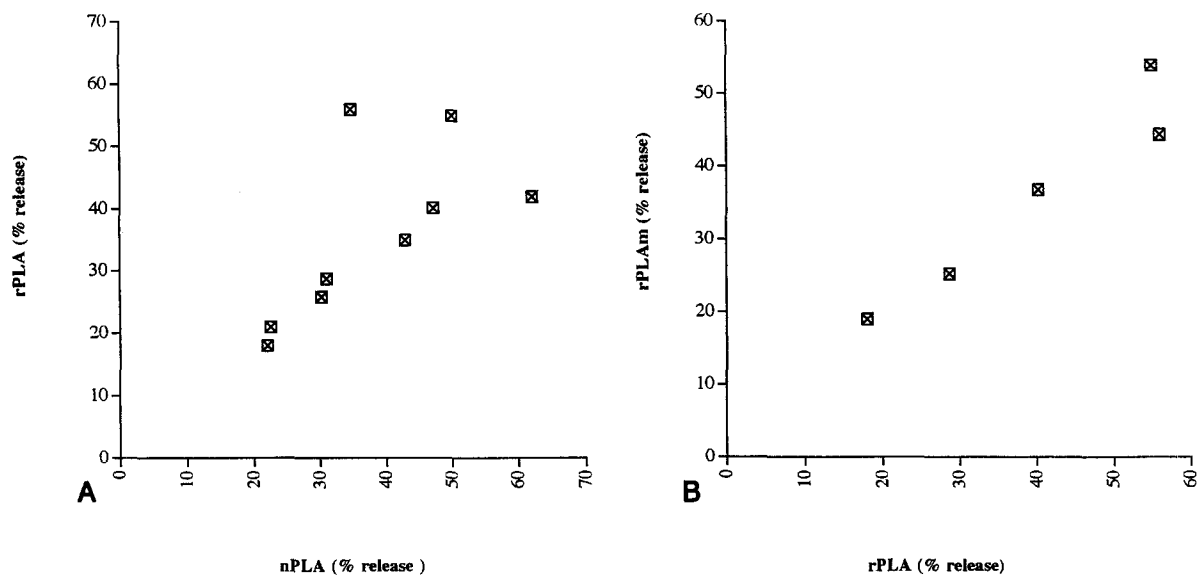


FIG. 4. Comparison of HR from nine individuals with 100 ng/ml of either natural PLA and rPLA (**A**) or rPLA and rPLAm (**B**).

not shown), indicating that a native-like three-dimensional structure was essential for IgE-dependent mediator release and thus for IgE recognition of antigen.

DISCUSSION

In the past, crude allergen extracts or purified allergen preparations have been used for in vitro diagnosis, skin testing, and hyposensitization of

patients with allergy. The major drawback of these preparations lies either in the presence of a bulk of irrelevant proteins or the potential loss of important allergens or their biologic activity during purification procedures.^{3,19} The introduction of recombinant DNA technology for the production of pure allergens is a promising step to circumvent these problems. However, to be of diagnostic or therapeutic use, recombinant allergens must have biologic activities similar to those of natural allergens.

Using bee venom allergy as an example to verify the diagnostic usefulness of a recombinant allergen, we took advantage of the availability of highly purified natural PLA in combination with the recently described biologically fully active recombinant enzyme.^{8,9} The data presented in this report show that there is no significant difference between natural and recombinant preparations in HR assays. Hence, each of the preparations could equally well be used to assist in the diagnosis of bee venom allergy. We further show that PLA can be genetically engineered to avoid undesired properties, such as enzymatic activity, without loss of allergenic potency in HR assays or in skin tests, as previously shown.¹²

Sugar residues on PLA have been implicated as dominant B-cell epitopes for IgE by means of serologic assays,²⁰ and hence these epitopes may be important in vitro in HR assays or in vivo in skin test assays. This would be a major drawback of recombinant allergen produced in bacteria, because prokaryotes do not glycosylate proteins. Indeed, in previous studies we noted that antibodies of some individuals allergic to bee venom also recognized sugar residues when their sera were analyzed in vitro.¹⁰ In addition, sugar residues may be a reason for slight individual differences observed when recombinant and natural PLA are compared in a quantitative skin test.^{12,21} However, in skin tests with rPLA or natural PLA, none of the 21 patients with bee venom allergy had negative results when challenged with either allergen preparation. Therefore neither the data analyzing PLA in vivo nor the data presented in this report provide any firm evidence that sugar residues are dominant or essential B-cell epitopes for IgE.

In previous studies 58 patients with bee venom allergy also showed type I skin reactivity to natural PLA, indicating that PLA is a major allergen in bee venom.²¹ This will provide a unique opportunity to analyze a single defined allergen available as natural or recombinant molecule and to compare its value with that of bee venom both in vivo and in

vitro for the routine diagnosis of Hymenoptera allergies.

We thank Dr. Ueli R. Müller (Zieglerspital) and Bern and Theres Schneider (SIAF, Davos) for helpful discussion and suggestions for the manuscript. It was a pleasure to informally discuss the data with Dr. Michael H. Gelb, University of Washington, Seattle and Dr. Birgit Helm (University of Sheffield, Sheffield, U.K.) in the mountain chalet graciously provided by the Andreas Valär family, Davos.

REFERENCES

1. Björkstén F. Standardisation of allergenic extracts of specific foods: apples. In: Schäffer M, Sisk C, Brede HD, eds. Regulatory control and standardisation of allergenic extracts. Fourth International Paul Ehrlich Seminar, Stuttgart, Germany: Gustav Fischer Verlag, 1987:279-84.
2. Gumowski PI, Latge JP, Paris D. Fungal allergy. In: Arora DK, ed. Handbook of applied mycology in humans, animals and insects. New York: Marcel Dekker Inc, 1991:53-103.
3. Kraft D, Schon A, eds. Molecular biology and immunology of allergens. Boca Raton, Florida: CRC press, 1993.
4. Habermann E. Bee and wasp venoms. *Science* 1972;177:314.
5. King TP, Sobotka AK, Kochoumian L, Lichtenstein LM. Allergens of honey bee venom. *Arch Biochem Biophys* 1976;172:661-71.
6. Müller UR. Insect sting allergy: clinical picture, diagnosis and treatment. Stuttgart, Germany: Gustav Fischer Verlag, 1990.
7. Kuchler K, Gmachl M, Sippl MJ, Kreil G. Analysis of the cDNA for phospholipase A₂ from honeybee venom glands. *Eur J Biochem* 1989;184:249-54.
8. Dudler T, Chen W-Q, Wang S, et al. High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A₂. *Biochim Biophys Acta* 1992;1165:201-10.
9. Dudler T, Schneider T, Annand RR, Gelb MH, Suter M. Antigenic surface of the bee venom allergen phospholipase A₂. Structural and functional analysis of human IgG₄ antibodies reveals potential role in protection. *J Immunol* 1994;152:5514-22.
10. Schneider T, Lang A, Carballido J, et al. Human monoclonal or polyclonal antibodies recognize predominantly discontinuous epitopes on bee venom phospholipase A₂. *J ALLERGY CLIN IMMUNOL* 1994;94:61-70.
11. Carballido JM, Carballido-Perrig N, Kägi MK, et al. T cell epitope specificity in human allergic and non-allergic individuals to bee venom phospholipase A₂. *J Immunol* 1993;150:3582-91.
12. Suter M, Dudler T, Schneider T, et al. Rekombinantes Bienengift-Allergen Phospholipase A₂: ein Modell für die Standardisierung von rekombinanten Allergenen. *Allergologie* 1994;9:414-8.
13. Goding JW. Monoclonal antibodies: principles and practice. New York: Academic Press, 1983.
14. Stüber D, Matile H, Garotta G. System for high level production in *E. coli* and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies and structure-function analysis. In: Lefkovits I, Pernis B, eds. Immunological methods. New York: Academic Press, 1990;4:121-52.

15. Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stüber D. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate absorbent. *Biotechnology* 1988;6:1321-5.
16. Siraganian RP. Histamine secretion from mast cells and basophils. *Trends Biol Sci* 1983;4:432-7.
17. Morel AM, Delaage MA. Immunoanalysis of histamine by a novel chemical derivatization. *J ALLERGY CLIN IMMUNOL* 1988;82:646-56.
18. MacGlashan DW Jr. Releasability of human basophils: cellular sensitivity and maximal histamine release are independent variables. *J ALLERGY CLIN IMMUNOL* 1993;91:605-615.
19. Ford SA, Baldo BA, Panzani R, Bass D. Cypress (*Cypressus sempervirens*) pollen allergens: identification by protein blotting and improved detection of specific IgE antibodies. *Int Arch Allergy Appl Immunol* 1991;95:178-83.
20. Weber A, Schröder H, Thalberg K, März L. Specific interaction of IgE antibodies with a carbohydrate epitope of honey bee venom phospholipase A₂. *Allergy* 1987;42:464-70.
21. Müller U, Dudler T, Schneider T, et al. Type I skin reactivity to native and recombinant phospholipase A₂ from honeybee venom is similar. *J ALLERGY CLIN IMMUNOL* (in press).

Bound volumes available to subscribers

Bound volumes of THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY are available to subscribers (only) for the 1995 issues from the Publisher, at a cost of \$73.00 for domestic, \$99.51 for Canadian, and \$93.00 for international subscribers for Vol. 95 (January-June) and Vol. 96 (July-December). Shipping charges are included. Each bound volume contains a subject and author index, and all advertising is removed. Copies are shipped within 30 days after publication of the last issue in the volume. The binding is durable buckram with the journal name, volume number, and year stamped in gold on the spine. *Payment must accompany all orders.* Contact Mosby-Year Book, Inc., Subscription Services, 11830 Westline Industrial Dr., St. Louis, MO 63146-3318; phone 1 (800) 453-4351 or (314) 453-4351.

Subscriptions must be in force to qualify. Bound volumes are not available in place of a regular journal subscription.