

M. Okano
K. Nishizaki
A.R. Satoskar
T. Yoshino
Y. Masuda
D.A. Harn Jr

Original article

Involvement of carbohydrate on phospholipase A₂, a bee-venom allergen, in *in vivo* antigen-specific IgE synthesis in mice

Authors' affiliations:

M. Okano, A.R. Satoskar, D.A. Harn Jr,
Department of Immunology and Infectious
Diseases, Harvard School of Public Health,
Boston, MA, USA

K. Nishizaki, M. Okano, Y. Masuda, Department
of Otolaryngology, Okayama University Medical
School, Okayama, Japan

T. Yoshino, Second Department of Pathology,
Okayama University Medical School, Okayama,
Japan

Correspondence to:

Mitsuhiko Okano, MD, PhD
Department of Otolaryngology
Okayama University Medical School
2-5-1 Shikata-cho
Okayama
700-8558
Japan

Date:

Accepted for publication 8 February 1999

To cite this article:

Okano M., Nishizaki K., Satoskar A.R., Yoshino T.,
Masuda Y. & Harn D.A. Jr. Involvement of carbohydrate
on phospholipase A₂, a bee-venom allergen, in *in vivo*
antigen-specific IgE synthesis in mice.
Allergy 1999; **54**: 811–818.

Copyright © Munksgaard 1999

ISSN 0105-4538

Key words: carbohydrate; IgE; intranasal sensitization;
phospholipase A₂.

Background: Carbohydrates on allergens are known to be important for allergenicity. However, most findings have been made with epitope analysis. In this study, we investigated the involvement of *N*-glycan on phospholipase A₂ (PLA₂), the major allergen of honeybee venom, in *in vivo* synthesis of specific IgE in mice.

Methods: CBA/J and C57BL/6 mice were sensitized intranasally with either native or deglycosylated PLA₂ in the absence of adjuvant. After repeated sensitization, serum Ab titers against PLA₂ were determined. PLA₂ was deglycosylated chemically with anhydrous trifluoromethanesulfonic acid (TFMS).

Results: CBA/J mice showed PLA₂-specific IgE production after repeated sensitization with native PLA₂. They also produced PLA₂-specific IgG1 predominantly, suggesting that Th2-type Ab production was induced. When we used deglycosylated PLA₂ as a competitor in ELISA for detecting PLA₂-specific IgE, deglycosylated PLA₂ completely inhibited the binding between native PLA₂ and IgE. Deglycosylated PLA₂ had the same potential for inducing specific IgE synthesis as native PLA₂, since sensitization with deglycosylated PLA₂ also elicited IgE production in CBA/J mice.

Conclusions: These results suggest that carbohydrate on PLA₂ is less important than previously thought not only as a dominant IgE epitope but also in synthesis of PLA₂-specific IgE *in vivo*.

Abbreviations. PLA₂: phospholipase A₂; TFMS: anhydrous trifluoromethanesulfonic acid; Ab: antibody; mAb: monoclonal antibody; Ag: antigen; Ig: immunoglobulin; SEM: standard error of mean.

Carbohydrates on glycoproteins have been known to have several immunoreactive functions. First, some glycans function as immunoglobulin epitopes to activate B cells (1, 2), and contribute to the cross-reactivity among glycosylated allergens having unrelated protein portions (3, 4). In addition, B-1 cells, the B-cell subsets that express CD5 molecule on their surface, are known to produce immunoglobulins or cytokines preferentially in response to pathogen-expressed polysaccharides (5). Second, these glycans regulate T-cell differentiation and activation (6–8). Third, cell-surface carbohydrates, especially sialyl Lewis^x and sialyl Lewis^a, are known to function as ligands for selectins and work as a first step in the traffic signals that regulate lymphocyte recirculation and leukocyte emigration (9, 10). Therefore, it is of interest to analyze the functional characterization of glycans on glycosylated allergens, which cause typical Th2 allergic reactions such as those of asthma, allergic rhinitis, atopic dermatitis, and lethal anaphylactic shock.

Phospholipase A₂ (PLA₂), which is considered to be the most allergenic component of the venom of the honeybee, *Apis mellifera*, often causes life-threatening, IgE-mediated anaphylactic allergic reactions in man (11). Although native PLA₂ contains an unglycosylated variant, it is a glycoprotein composed of 134 amino acids and has a single N-glycosylation site linked to Asp 13 (12). In addition, in the primary structures of the N-linked carbohydrate chains of PLA₂, it was found that the oligosaccharides consisted of mannose, N-acetylglucosamine, and fucose α 1-6 and/or α 1-3 linked to the innermost N-acetylglucosamine (13, 14).

An issue that has remained controversial is the ability of N-glycan of PLA₂ to be an epitope for PLA₂-specific IgE (15–19). Moreover, it remains unclear whether the N-glycan can elicit PLA₂-specific IgE production. To investigate the induction of antigen-specific IgE *in vivo*, we must develop predictive animal models. PLA₂ has previously been shown to induce IgE and IgG responses in mice (20–22). However, in all the reports reviewed, PLA₂ was inoculated together with adjuvants. Adjuvants are known to modulate the immune response nonspecifically (23, 24). In addition, some adjuvants are known to denature the native Ag, and denatured PLA₂ lacks the ability to induce significant histamine release by basophils obtained from bee-venom allergics (18, 26). Therefore, in an investigation of antigenicity, it is desirable to use Ag in the absence of adjuvants (27–30). In this study, we established a murine model that induced a dominant Th2-type Ab production following repeated intranasal sensitization with PLA₂ in the absence of adjuvants. And we investigated whether the carbohydrate

of PLA₂ is critical for the induction of antigen-specific IgE production and the recognition of PLA₂ by IgE in this model.

Material and methods

Animals

Young adult (7–10 weeks old) CBA/J and C57BL/6 strain female mice were purchased from Harlan (Indianapolis, IN, USA). The mice were maintained at the Harvard School of Public Health according to the guidelines set forth by the Harvard Medical Area Research Committee.

Reagents

Purified rat anti-mouse IgE mAb was purchased from Biosource (Camarillo, CA, USA); mouse IgE mAb and biotinylated rat anti-mouse IgE mAb from Pharmingen (San Diego, CA, USA); PLA₂, mouse IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgM, and IgA mAbs, and extravidin-peroxidase conjugate from Sigma Chemical Co. (St Louis, MO, USA); goat anti-mouse IgG, IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ mAb-peroxidase conjugate from Boehringer-Mannheim (Indianapolis, IN, USA); Tween 20 from Bio-Rad (Hercules, CA, USA); tetramethylbenzidine substrate for peroxidase from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA); and FCS from Gibco BRL (Grand Island, NY, USA). For biotinylation, PLA₂ (1.8 mg/ml) in sodium bicarbonate buffer, pH 8.5, was incubated with biotin (long-arm) N-hydroxy succinimide ester (Vector Lab., Burlingame, CA, USA) for 2 h at room temperature. The reaction was stopped by adding 5 μ l of ethanolamine (Sigma), and dialyzed overnight with PBS containing 0.05% sodium azide. The protein assay was performed by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Sensitization of mice

Mice ($n=5$) were sensitized with 20 μ l of either 0.1 μ g (low) or 10 μ g (high) PLA₂ concentrated with Dulbecco's PBS (Gibco) through both nostrils with a microsyringe (Hamilton Co., Reno, NV, USA). Two weeks later, this treatment was repeated in the same fashion. From day 21, the same amount of Ag was inoculated for 7 consecutive days. This procedure was termed the nasal challenge. Blood samples were taken from tail veins 9 (after priming), 20 (after boosting), and 27 days (after challenge) after primary sensitization.

Serum total IgE

Serum total IgE was measured by sandwich ELISA. ELISA plates (Corning, Inc., Corning, NY, USA) were coated overnight at 4°C with 5 µg/ml rat anti-mouse IgE mAb in 0.05 M carbonate buffer, pH 9.6. Plates were then blocked for 2 h at 37°C with 200 µl of PBS containing 10% FCS and 0.3% Tween 20. After blocking, the serum samples or standard mouse IgE mAb in serial dilutions with PBS containing 10% FCS were added, and the plates were incubated for 2 h at 37°C. Thereafter, the plates were incubated with 500 ng/ml biotinylated rat anti-mouse IgE mAb for 2 h at 37°C, and next with 1/1000 extravidin-peroxidase conjugate for 1 h at 37°C, and then substrate was added. Finally, 5% phosphoric acid (Fisher Scientific, Pittsburgh, PA, USA) was added to stop the reaction, and the absorbance at 450 nm was measured with an automatic microplate reader (Molecular Devices, Menlo Park, CA, USA). The amount of total IgE in ng/ml was analyzed by the Softmax software program. Between each step, plates were washed four times (except the final wash which was done eight times) with PBS containing 0.05% Tween 20.

PLA2-specific IgE

Antigen-specific IgE was also determined by sandwich ELISA using biotinylated antigen as a detection reagent. ELISA plates were coated and blocked in the same way for the measurement of serum total IgE. After the incubation of diluted samples for 2 h at 37°C, biotinylated PLA2 (1 µg/ml) was added to the plates for 2 h at 37°C, followed by extravidin-peroxidase conjugate for 1 h at 37°C. Finally, the plates were developed with substrate and stopped with 5% phosphoric acid. The plates were read in a microplate reader, as described above.

PLA2-specific IgG

Antigen-specific IgG Ab was detected by indirect ELISA. ELISA plates were coated with PLA2 (2 µg/ml) overnight at 4°C, and blocked as described above. Each individual serum sample was then plated in duplicate in twofold serial dilution, beginning at 1:100, and incubated for 2 h at 37°C. Thereafter, the plates were incubated with goat anti-mouse IgG mAb-peroxidase conjugate (1/10000) for 1 h at 37°C. The plates were developed with substrate, and stop solution was added. Finally, the OD value was determined at 450 nm by a microplate reader. For the detection of antigen-specific Ig subclasses, 1/2000 goat anti-

mouse IgG1, IgG2a, IgG2b, or IgG3 mAb-peroxidase conjugate was used instead of goat anti-mouse IgG mAb-peroxidase conjugate. Results are expressed as end-point titers where the end-point equals the final serum dilution yielding an absorbance twice that of background.

Inhibition ELISA

To determine whether the glycan of PLA2 can function as an IgE epitope, we performed competitive inhibition ELISA. ELISA plates were coated overnight with 5 µg/ml rat anti-mouse IgE mAb. After blocking, 1:6 diluted pooled sera from CBA/J mice challenged with PLA2 were added in triplicate wells. Then, biotinylated PLA2 (1 µg/ml) mixed with deglycosylated PLA2 in serial concentrations (0, 0.1, 1, 10, and 100 µg/ml) was added to the plates, followed by 1:1000 extravidin-peroxidase conjugate. Finally, the plates were developed with substrate and stopped with 5% phosphoric acid. The plates were read in a microplate reader as described above.

Deglycosylation of PLA2

PLA2 was deglycosylated chemically with the Glyco Free[™] deglycosylation kit (Oxford Glycosystems, Bedford, MA, USA) according to the manufacturer's instructions. Two methods were used to confirm deglycosylation. First, SDS-PAGE (15%) was performed by the method of Laemmli under reducing conditions (31). Approximately 5 µg of PLA2 or trifluoromethanesulfonic acid (TFMS)-treated PLA2 was loaded per lane, and then stained with Coomassie brilliant blue to detect precipitated protein (Pierce). Second, a binding assay of lectin against PLA2 was analyzed. ELISA plates were incubated overnight with 50 µl/well native or TFMS-treated PLA2 (2 µg/ml) diluted in carbonate buffer. The plates were washed and blocked with PBS containing 0.3% Tween 20 for 1 h at 37°C. Biotinylated lectin serially diluted with lectin-binding (LB) buffer (150 mM NaCl, 0.2 mM CaCl₂, 0.1 mM MnSO₄, 0.1 mM MgCl₂, 1 mM HEPES, and 0.3% Tween 20) was added for 1 h at 37°C. Unbound lectin was removed by washing with LB buffer, and the wells were incubated with peroxidase-conjugated extravidin diluted 1:1000 in LB buffer for 1 h at 37°C. The plates were again washed with LB buffer, and the bound peroxidase was measured by adding TMB substrate and reading at 450 nm on a microplate reader. Biotinylated lectin used in this study was Galanthus Nivialis Lectin (GNL, Vector), which binds to α-linked mannose residues.

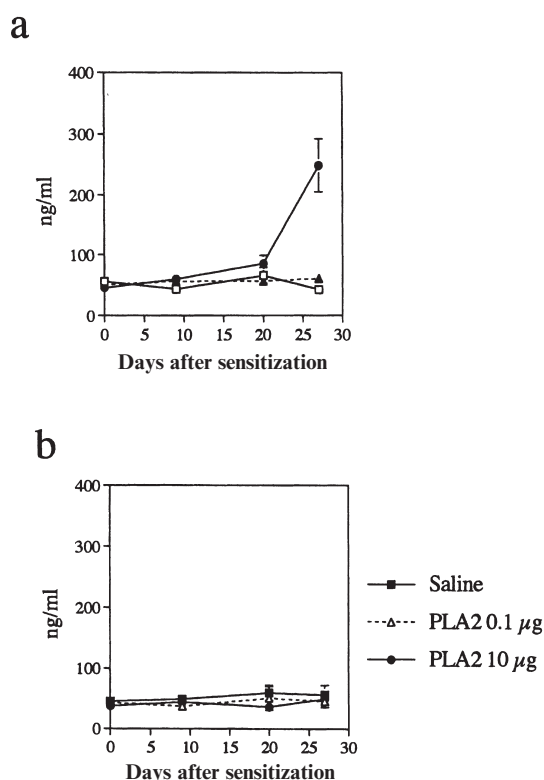


Figure 1. Amount of serum total IgE in mice after intranasal sensitization with PLA2. CBA/J a) and C57BL/6 b) mice received 10 µg PLA2 (circle), 0.1 µg PLA2 (triangle), or saline (square) in absence of adjuvant. Two weeks later, this treatment was repeated in same fashion. From day 21, same amount of Ag was inoculated for 7 consecutive days; this we termed “nasal challenge”. Sera were taken from tail vein 9, 20, and 27 days after primary sensitization. Results show mean concentration $\pm$ SEM of pooled sera from five mice. Data represent two separate experiments.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of mean (SEM) for each subject group. Statistical analysis was performed with Student's unpaired *t*-test to compare different groups of mice. A value of $P < 0.05$ was considered significant.

Results

Serum total IgE after repeated intranasal sensitization with PLA2

Two different strains of mice were sensitized intranasally with low (0.1 µg) or high (10 µg) doses of PLA2 in the absence of adjuvants. Only CBA/J mice showed an increased amount of serum IgE in response to a high dose of PLA2 after the nasal challenge (Fig. 1a). C57BL/6 mice did not show elevation of IgE after sensitization (Fig. 1b). Levels of

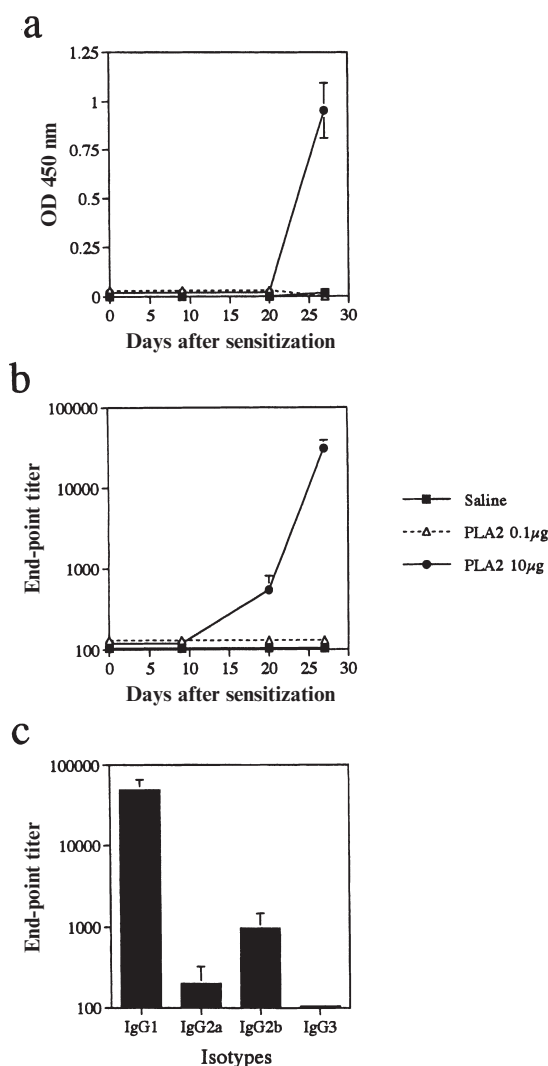


Figure 2. Serum levels of PLA2-specific IgE a) and IgG b). CBA/J mice were sensitized intranasally, as described in Fig. 1. Titers for PLA2-specific IgE and IgG are shown as mean OD at 450 nm of 1:4 diluted sera (a) and end-point titer (b) $\pm$ SEM of pooled sera from five mice, respectively. c) IgG isotypes specific for PLA2. Results show mean end-point titer $\pm$ SEM from five CBA/J mice after nasal challenge. Data represent two separate experiments.

normal serum IgE were 44.74 ± 7.51 ng/ml for CBA/J mice and 42.21 ± 14.49 for C57BL/6 mice, respectively ($n=6$). In this ELISA assay, the lower detection limit was 1.0 ng/ml, and mouse IgG, IgM, and IgA did not respond to this IgE ELISA system (data not shown).

Antigen-specific IgE and IgG production after sensitization with PLA2

As well as the increase of total serum IgE, only CBA/J mice sensitized with 10 µg of PLA2 produced a significant amount of PLA2-specific IgE after the nasal challenge (Fig. 2a). This signal was completely diminished when sera were heated at

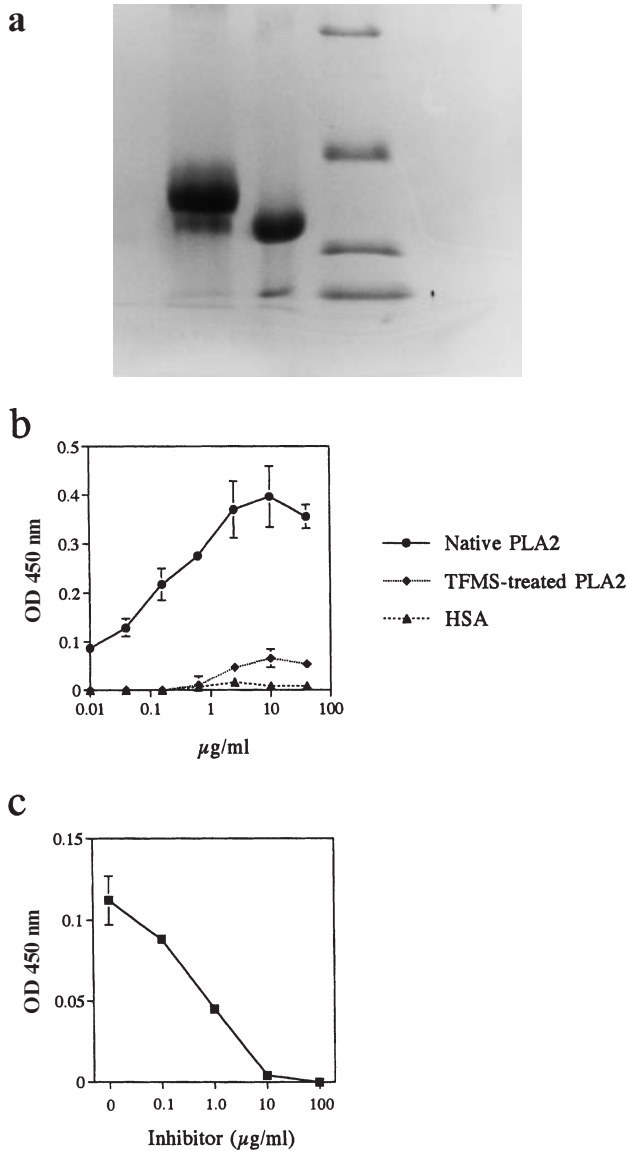


Figure 3. a) SDS-PAGE of native PLA2 (left lane) and TFMS-treated PLA2 (middle lane). Molecular mass marker (6.5, 14.5, 21.5, and 31.0 kDa) was loaded in right lane. b) Binding ability of native and TFMS-treated PLA2 to GNL lectin. Naive PLA2 (circle), TFMS-treated PLA2 (diamond), or human serum albumin (HSA, triangle) as unrelated Ag was examined for binding ability to serially diluted GNL lectin, as described in Material and methods. Results show mean OD value at 450 nm $\pm$ SEM of triplicated wells. c) Inhibition ELISA using deglycosylated PLA2 as competitor for biotinylated PLA2. Biotinylated PLA2 (1 µg/ml) was mixed with deglycosylated PLA2 in serial concentrations (0, 0.1, 1, 10, and 100 µg/ml). Results show mean OD at 450 nm $\pm$ SEM of 1:6 diluted pooled sera from five mice intranasally sensitized with PLA2. Data represent two separate experiments.

56°C for 1 h (data not shown). On the other hand, PLA2-specific IgG was also detected after a second sensitization with 10 µg of PLA2 in CBA/J mice (Fig. 2b). PLA2-specific IgG1 was the predominant isotype of the IgG isotypes, suggesting that this strain produced Th2-type Ab (Fig. 2c). In

contrast, C57BL/6 mice did not produce detectable levels of Ag-specific IgE or IgG after intranasal sensitization with either 0.1 or 10 µg of PLA2 (data not shown).

Carbohydrate motif on PLA2 for IgE epitope

To determine whether the carbohydrate motif on PLA2 could be recognized by PLA2-specific IgE, we tested inhibition ELISA, using deglycosylated PLA2 as a competitor for biotinylated PLA2. PLA2 was deglycosylated chemically with TFMS. Deglycosylation was confirmed by SDS-PAGE (Fig. 3a). Native PLA2 showed a molecular mass band at approximately 16 kDa, and TFMS-treated PLA2 reduced the molecular mass by approximately 3 kDa; this was equivalent to the carbohydrate motif reported (13, 14). Moreover, in the lectin-binding assay, TFMS-treated PLA2 reduced the binding activity against 10 µg/ml GNL lectin to 83.38% compared to native PLA2, suggesting that mannose was lost from native PLA2 by TFMS treatment (Fig. 3b). Using this deglycosylated PLA2 as a competitive Ag, we performed inhibition ELISA to detect PLA2-specific IgE Ab. As shown in Fig. 3c, deglycosylated PLA2 inhibited the absorbance of PLA2-specific IgE in a dose-dependent manner, and eliminated it at the concentration of 10 µg/ml. This result suggested that the carbohydrate on PLA2 was less important for the recognition of PLA2 by specific IgE.

Ab production after sensitization with deglycosylated PLA2

To investigate whether the carbohydrate motif on PLA2 could induce IgE production in responder mice, we sensitized CBA/J mice with deglycosylated PLA2. When we sensitized them with 0.1 µg of deglycosylated PLA2, they did not produce PLA2-specific IgE (data not shown). On the other hand, both the increase of serum total IgE and the induction of PLA2-specific IgE could be seen in mice after repeated intranasal sensitization with 10 µg of deglycosylated PLA2 (Fig. 4a and b). The amount of specific IgE was not statistically different between mice receiving native and deglycosylated PLA2. In addition, the absorbance of PLA2-specific IgE in mice sensitized with deglycosylated PLA2 was inhibited by both native and deglycosylated PLA2 at the same magnitude, suggesting that the chemical deglycosylation step had no effect on altering the secondary or tertiary structure of PLA2 and creating new antigenic epitopes on this molecule (data not shown). The same finding was seen regarding PLA2-specific IgG. After the nasal challenge, PLA2-specific IgG1 isotype was produced predominantly in mice sensitized with both deglycosylated and native PLA2

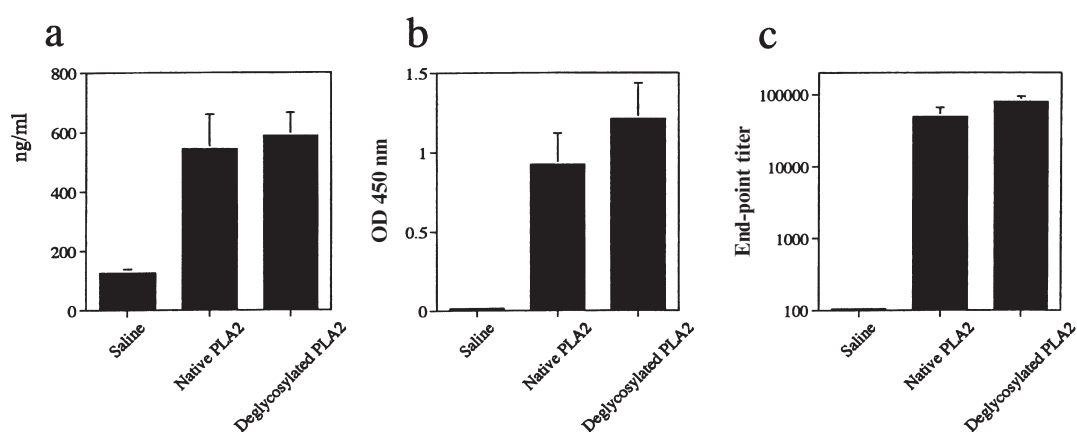


Figure 4. Ab production in mice sensitized with deglycosylated PLA2. CBA/J female mice were sensitized with saline, or 10 μ g native or deglycosylated PLA2. After nasal challenge, sera were taken, and serum total IgE (a), PLA2-specific IgE (b), and PLA2-specific IgG1 (c) were determined. Results show mean concentration (a), OD at 450 nm of 1:4 diluted sera (b), and end-point titer (c) $\pm$ SEM of pooled sera of five mice for serum total IgE, PLA2-specific IgE, and PLA2-specific IgG1, respectively. Data represent two separate experiments.

at the same magnitude (Fig. 4c). In this regard, deglycosylated PLA2 had the same potential for inducing antigen-specific IgE and IgG1 production, suggesting that the carbohydrate portion in PLA2 did not seem necessary to induce Th2-type Ab production.

Discussion

The present study demonstrates that intranasal sensitization of PLA2 induced not only the increase of serum total IgE but also the production of PLA2-specific IgE and IgG in the absence of adjuvants in CBA/J mice. CBA/J mice produced high amounts of IgG1 compared with the other IgG isotypes. In mice, induction of IgG1 isotype is mediated by the Th2 immune response (32). Consideration of these results as a whole suggests that this strain produced Th2-type Ab in response to PLA2.

In combination with several adjuvants, PLA2 is known to elicit IgE and IgG responses in mice. Kolbe et al. reported that CBA/J mice produced a high level of PLA2-specific IgE after intraperitoneal injection with 0.1 μ g of PLA2 adsorbed to 2 mg Al(OH)₃, whereas those immunized with 10 μ g of PLA2 had a low, transient level of PLA2-specific IgE (20, 21). Germann et al. reported that CBA/J mice intraperitoneally immunized six times at biweekly intervals with 0.1 μ g or three times with 5 μ g of PLA2 adsorbed to 2 mg Al(OH)₃ produced PLA2-specific IgE (22). Our results are consistent with these results regarding the responsible strain of mice against PLA2. Lucas & Hamburger found that the murine Ab response to PLA2 is under the control of genes in the H-2 complex (33). They suggested that mice with the H-2b

(C57BL/6) haplotype responded poorly, while those with the H-2d, H-2k, H-2q, and H-2s haplotypes responded by producing specific Ab. This report may support our finding that the CBA/J strain is a high responder, whereas C57BL/6 is a nonresponder in intranasal sensitization with PLA2.

On the other hand, our results for the dose of the Ag used for sensitization differ from the reports mentioned above (20–22). The dose of Ag is very important for controlling differential activation of particular Th responses. Hosken et al. suggested that midrange doses of antigenic peptide direct the development of Th1 cells, whereas very high and very low Ag doses induce Th2 cells (34). This difference may have arisen from the use of adjuvants and/or the route of sensitization. First, adjuvants are known to augment a particular differentiation toward Th1 or Th2 responses, and the use of adjuvants requires great care in the modulating of immune responses. Aluminum hydroxide (alum, Al(OH)₃) and diesel-exhaust particulates are known to act as adjuvants to induce Th2 responses preferentially in rodents (23, 25, 30). Thus, the use of adjuvants makes the difference in the optimal dose of the ability of Ag to induce an immune reaction. Therefore, the absence of adjuvants is desirable for investigating the physical development of immune responses to Ag that reflect natural exposure. Second, the route of immunization is critical for developing the differentiation into either Th1 or Th2 type responses. Xu-Amano et al. demonstrated that oral immunization of mice generated predominantly Th2 cells in Peyer's patches and the spleen, whereas systemic immunization generated both Th1 and Th2 cells (35). It is known that intranasal or aerosolized immunization with Ag preferentially induces IgE production in the absence of adjuvants (28–30), perhaps

because of the existence of dendritic cells in the respiratory tract possessing a strong function of Ag presentation (36). Thus, it is possible to speculate that IgE production against external Ag is liable to be induced in an airway route which is one of the first lines of defense against the external environment.

Using this model, we revealed that the carbohydrate on PLA2 is less critical as an IgE epitope. There are contrary findings in the role of *N*-glycan of PLA2 for recognition of PLA2-specific IgE from both man and animal models. Weber et al. reported that an oligosaccharide found in PLA2 could represent an epitope which is recognized by IgE in 5/11 patients allergic to PLA2 (15). Tretter et al. also reported that 34/122 sera from honeybee-allergic patients exhibited significant amounts of glycan-reactive IgE, and demonstrated that α 1,3-fucosylation of the innermost *N*-acetylglucosamine residue of *N*-glycoproteins forms an IgE-reactive determinant (16). Prenner et al. identified α 1,3-fucosylation of the asparagine-bound *N*-acetylglucosamine on PLA2 as the antigenic determinant in a rabbit polyclonal antiserum against native PLA2 (17). In contrast, Forster et al. reported that recombinant enzymatically active PLA2 and purified natural protein were equally effective in releasing histamine from sensitized basophils in patients with Hymenoptera allergy, suggesting that sugar residues are not dominant or essential B-cell epitopes for IgE (18). Müller et al. also reported that native PLA2 and deglycosylated native PLA2 resulted in similar skin reaction, indicating that the sugar residues were of little relevance to IgE-binding in the patients (19). Schneider et al. demonstrated that human mAbs against native PLA2 also recognized deglycosylated PLA2 to the same extent, suggesting that sugar residues did not contain dominant B-cell epitopes on PLA2 (37). Our results are consistent with those of the last named.

Our results show that the *N*-glycan of PLA2 is less important for IgE production in a murine model of intranasal sensitization. In general, IgE production is under the control of IL-4-producing T cells (38), and we reported that CD4+ T cells reactive to house-dust mite isolated from allergic

individuals produced high amounts of IL-4 and IL-5 in response to purified mite allergen, whereas those from sensitized healthy subjects induced high levels of IFN- γ in response to the same allergen under the restriction of the identical HLA-DR allele (39). Recently, in a human *in vitro* system, carbohydrate-dependent, HLA-class II-restricted T-cell clones specific for PLA2 were generated, and this demonstrated that the mannose-containing end of the carbohydrate moiety may be required for epitope recognition of processed PLA2 by the HLA-class II desotope (7). Our results are not consistent with the report. As they show, not all the allergics or T-cell clones specific to PLA2 showed a T-cell response to the carbohydrate motif on PLA2. Thus, the immune responses to PLA2 may be a result of the heterogeneity, and CBA/J mice are the responder strain to PLA2 and recognize the peptide portion of the Ag. *In vitro* findings might also reflect a minor response of the immune system to glycoproteins. Taking these findings together, it is tempting to speculate that the ability of sugar chains to promote IgE production depends on the compositions and locations of sugar residues and/or the nature of the antigenicity of peptides themselves. Further work into what especially makes PLA2 antigenic in the absence of adjuvants, e.g., processing pathways, would be of great interest.

In conclusion, we generated a murine intranasal sensitization model specific for PLA2 in the absence of adjuvant. Using this model, we demonstrated that the carbohydrate of PLA2 is less important for the promotion or recognition of IgE specific for this Ag. These findings may contribute to the future diagnosis of this allergy and immunotherapy using recombinant peptide/allergens that do not include sugar chains.

Acknowledgments We thank Mie Abe and Mao-Mao Wang for technical assistance, and Joan Sawyer for help in preparing the manuscript. This work was supported by Public Health grant AI-16305-18 from the National Institutes of Health (USA).

References

1. Kurosaka A, Yano A, Itoh N, Kuroda Y, Nakagawa T, Kawasaki T. The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J Biol Chem* 1991;**266**:4168–4172.
2. Ohta M, Matsuura F, Kobayashi Y, Shigeta S, Ono K, Oka S. Further characterization of allergenically active oligosaccharitols isolated from a sea squirt H-antigen. *Arch Biochem Biophys* 1991;**290**:474–483.
3. Hijikata A, Matsumoto I, Kojima K, Ogawa H. Antigenicity of the oligosaccharide moiety of the Japanese cedar (*Cryptomeria japonica*) pollen allergen, *Cry j I*. *Int Arch Allergy Immunol* 1994;**105**:198–202.

4. Batanero E, Villalba M, Monsalve RI, Rodríguez R. Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen. *J Allergy Clin Immunol* 1996;**97**:1264–1271.
5. Venupillai P, Secor WE, Horauf AM, Harn DA. B-1 cell (CD5+B220+) outgrowth in murine schistosomiasis is genetically restricted and is largely due to activation by polylactosamine sugars. *J Immunol* 1997;**158**:338–344.
6. Velupillai P, Harn DA. Oligosaccharide-specific induction of interleukin 10 production by B220+ cells from schistosome-infected mice: a mechanism for regulation of CD4+ T-cell subsets. *Proc Natl Acad Sci USA* 1994;**91**:18–22.
7. Dudler T, Altmann F, Carballido JM, Blaser K. Carbohydrate-dependent, HMC class II-restricted, human T cell response to bee venom allergen phospholipase A₂ in allergic patients. *Eur J Immunol* 1995;**25**:538–542.
8. Corinti S, Palma RD, Fontana A, Gagliardi C, Pini C, Sallusto F. Major histocompatibility complex-independent recognition of a distinctive pollen antigen, most likely a carbohydrate, by human CD8+ α/β T cells. *J Exp Med* 1997;**186**:899–908.
9. Lasky LA. Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science* 1992;**258**:964–969.
10. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994;**76**:301–314.
11. Reisman RE. Hymenoptera. *Immunol Allergy Clin North Am* 1995;**15**:567–573.
12. Kuchler K, Gmachl M, Sippl MJ, Kreil G. 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* 1989;**184**:249–254.
13. Staudacher E, Altmann F, Gloss J, et al. GDP-fucose: β -N-acetylglucosamine (Fuco (Fuc α 1-6-GluNAc)-Asn-peptide) α 1-3 fucosyltransferase activity in honeybee (*Apis mellifera*) venom glands. The difucosylation of asparagine-bound N-acetylglucosamine. *Eur J Biochem* 1991;**199**:745–751.
14. Kubelka V, Altmann F, Staudacher E, et al. Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A₂. *Eur J Biochem* 1993;**213**:1193–1204.
15. Weber A, Schroder H, Thalberg K, Marz L. Specific interaction of IgE antibodies with a carbohydrate epitope of honeybee venom phospholipase A₂. *Allergy* 1987;**42**:464–470.
16. Tretter V, Altmann F, Kubelka V, Marz L, Becker WM. Fucose α 1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. *Int Arch Allergy Immunol* 1993;**102**:259–266.
17. Prenner C, Mach L, Grossl J, Marz L. The antigenicity of the carbohydrate moiety of an insect glycoprotein, honey-bee (*Apis mellifera*) venom phospholipase A₂. The role of α 1,3-fucosylation of the asparagine-bound N-acetylglucosamine. *Biochem J* 1992;**284**:377–380.
18. Forster E, Duddler T, Gmachl M, Aberer W, Urbanek R, Suter M. 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. *J Allergy Clin Immunol* 1995;**95**:1229–1235.
19. Müller UR, Duddler T, Schneider T, et al. Type I skin reactivity to native and recombinant phospholipase A₂ from honeybee venom is similar. *J Allergy Clin Immunol* 1995;**96**:396–402.
20. Kolbe L, Heusser C, Kolsch E. Antigen-dose dependent regulation of B ϵ -memory cell expression. *Int Arch Allergy Appl Immunol* 1991;**95**:202–206.
21. Dudler T, Machado DC, Kolbe L, et al. A link between catalytic activity, IgE-independent mast cell activation, and allergenicity of bee venom phospholipase A₂. *J Immunol* 1995;**155**:2605–2613.
22. Germann T, Guckes S, Bongartz M, et al. Administration of IL-12 during ongoing immune responses fails to permanently suppress and can even enhance the synthesis of antigen-specific IgE. *Int Immunol* 1995;**7**:1649–1657.
23. Vijay HM, Lavergne G, Huang H, Bernstein IL. Preferential synthesis of IgE reaginic antibodies in rats immunized with alum-adsorbed antigens. *Int Arch Allergy Appl Immunol* 1979;**59**:227–232.
24. Audera C, Ramirez J, Soler E, Carreira J. Liposomes as carriers for allergy immunotherapy. *Clin Exp Allergy* 1991;**21**:139–144.
25. Aramaki Y, Suda H, Tsuchiya S. Interferon- γ inductive effect of liposomes as an immunoadjuvant. *Vaccine* 1995;**13**:1809–1814.
26. Scibienski RJ. Denaturation of lysozyme by Freund's complete adjuvant. *J Immunol* 1973;**111**:114–120.
27. Holt PG, Batty JE, Turner KJ. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 1981;**42**:409–417.
28. Renz H, Smith HR, Henson JE, Ray BS, Irvin CG, Gelfand EW. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J Allergy Clin Immunol* 1992;**89**:1127–1238.
29. Ahlstedt S, Björkstén B. Specific antibody responses in rats and mice after daily immunization without adjuvant. *Int Arch Allergy Appl Immunol* 1983;**71**:293–299.
30. Takafuji S, Suzuki S, Koizumi K, et al. Diesel-exhaust particulates inoculated by the intranasal route have an adjuvant activity for IgE production in mice. *J Allergy Clin Immunol* 1987;**79**:639–645.
31. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.
32. Finkelman FD, Holmes J, Katona IM, et al. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Annu Rev Immunol* 1990;**8**:303–333.
33. Lucas A, Hamburger RN. Immune response gene control of the murine antibody response to the phospholipase A₂ of honeybee venom. *Immunologist* 1986;**23**:417–420.
34. Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med* 1995;**182**:1579–1584.
35. Xu-Amano J, Aicher WK, Taguchi T, Kiyono H, McGhee JR. Selective induction of Th2 cells in murine Peyer's patches by oral immunization. *Int Immunol* 1992;**4**:433–445.
36. Nelson DJ, McMenamin C, McWilliam AS, Brenan M, Holt PG. Development of the airway intraepithelial dendritic cell network in the rat from class II major histocompatibility (Ia)-negative precursors: differential regulation of Ia expression at different levels of the respiratory tract. *J Exp Med* 1994;**179**:203–212.
37. Schneider T, Lang AB, Carballido JM, et al. Human monoclonal or polyclonal antibodies recognize predominantly discontinuous epitopes on bee venom phospholipase A₂. *J Allergy Clin Immunol* 1994;**94**:61–70.
38. Del Prete G, Maggi E, Parronchi P, et al. IL-4 is an essential factor for the IgE synthesis induced *in vitro* by human T cell clones and their supernatants. *J Immunol* 1988;**140**:4193–4198.
39. Okano M, Nagano T, Nakada M, et al. Epitope analysis of HLA-DR restricted helper T-cell responses to *Der p* II, a major allergen molecule of *Dermatophagoides pteronyssinus*. *Allergy* 1996;**51**:29–35.