

A Link Between Catalytic Activity, IgE-Independent Mast Cell Activation, and Allergenicity of Bee Venom Phospholipase A₂¹

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The molecular and cellular mechanisms controlling Ab isotype selection following encounter of a given Ag are still unclear, although the regulatory role of cytokines is established. In the present study we explored the possibility that the nonimmunologic interaction of an allergen with cells of the innate immune system might result in a release of mediators that promote IgE isotype selection in adaptive responses. Using the bee venom allergen phospholipase A₂ (PLA₂) and a mutant variant lacking enzymatic function, we show that PLA₂, but not its catalytically inactive variant, is able to induce IgE-independent mediator release, including IL-4, from rodent mast cells. Assessing the in vivo relevance of these observations, we find that repeated injections of low doses of active enzyme into mice induce the synthesis of high levels of PLA₂-specific IgE, while immunization with the inactive form yields no detectable IgE response. Both Ags were similarly immunogenic when high doses of Ag were used for immunization. These findings suggest that mast cells might be a source of IL-4 at the onset of specific immunity against sources of allergens such as bee venom that contain PLA₂ and support the concept that the biologic action of an Ag on cells of the innate immune system can play a key role in determining adaptive immune responses. *The Journal of Immunology*, 1995, 155: 2605–2613.

In mammals, the exposure to certain Ags gives rise to a preferential synthesis of IgE Abs, suggesting that the isotype selection may be related to the nature of the Ag. The sustained production of IgE Abs as a result of

parasitic infestations is a well recognized and beneficial immune defense mechanism (1). In contrast, the induction of IgE synthesis by a large number of seemingly diverse and mainly innocuous Ags, including pollen grains, mold spores, house dust mite and cockroach emanations, insect venoms, and food components, is a pathologic immune response linked to the development of allergy and asthma (2). The reasons why these Ags cause abundant IgE Ab synthesis are poorly understood. The possibility that certain Ags share common structural motifs that selectively induce class switching to IgE has been investigated extensively, but despite intensive efforts and a considerable amount of information regarding the molecular structure of many common allergens, no unifying feature has emerged (2).

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In humans as well as in rodents, it is established that an elevated IgE Ab synthesis is associated with a Th cell response predominated by cytokine profiles of the Th2 phenotype (3, 4). The current concept is that Th2 and Th1 subsets differentiate from a common precursor, which has the capacity to secrete Th1- and Th2-type cytokines (5). Recent in vitro and in vivo studies have emphasized the pivotal importance of the cytokine environment present during the priming of these naive precursor T cells in determining their preferential differentiation into Th1 or Th2 subsets: IFN- γ and IL-12 drive Th1 differentiation, whereas IL-4 promotes Th2 development (6–8). This raises questions about the molecular and cellular mechanisms by which an Ag or invading pathogen can create such a cytokine environment at a time when specific lymphocyte responses are not yet established (5, 9).

Regarding the mechanisms leading to preferential Th1 responses following infections with intracellular parasites, it has been shown that macrophages infected with *Lysteria monocytogenes* are a source of IL-12, which subsequently directs Th1 development of naive CD4⁺ T cells (10, 11), and that this effect of IL-12 seems to be partially mediated by NK cells (7, 12).

Concerning Th2 responses, even though the role of IL-4 in priming naive T cells for subsequent Th2 differentiation is well established (13, 14), the cellular source of IL-4 following encounter of the allergen or helminth parasites is unclear. It has been speculated that mast cells or basophils might be the initial source of IL-4, and that the interaction of helminths or allergens with these cells may induce IL-4 release (8, 9).

We investigated this possibility using bee venom phospholipase A₂ (PLA₂),⁵ the main allergen involved in bee venom anaphylaxis (15–17). This Ag has previously been shown to elicit IgE and IgG Ab responses in mice and humans (15, 18), and its structural, functional, and immunologic characteristics have been studied extensively (19, 20). Since phospholipase A₂ enzymes have been shown to induce mediator release from mast cells (21), we reasoned that PLA₂ might trigger mast cells production of mediators such as IL-4 in an enzyme mediated mechanism. To test this hypothesis, we have generated a PLA₂ variant devoid of enzymatic activity by mutation of a catalytically conserved residue and have compared its ability to activate mast cells with its wild-type counterpart. We show that enzymatically active PLA₂, but not its catalytically inactive variant, induces nonimmunologic mediator release, including histamine and IL-4, from cells of rodent mast cell lineage. By comparing their immunogenicity in vivo, we further demonstrate that the catalytically inactive variant is unable to induce detectable IgE Ab synthesis under

conditions in which its wild-type counterpart induces maximal IgE responses. The findings indicate that the enzymatic function of PLA₂ profoundly affects its immunologic behavior and suggest that mast cells might play a prominent role in the initiation of IgE Ab responses against this allergen.

Materials and Methods

Reagents, enzymes, and general procedures

Chemicals of the highest available grade were purchased from Fluka (Buchs, Switzerland) or Sigma Chemical Co. (St. Louis, MO). DNA modifying enzymes were obtained from Boehringer Mannheim (Mannheim, Germany) and used as recommended. Plasmid DNA was prepared with anionic exchange columns (Qiagen kit, Qiagen Inc., Düsseldorf, Germany). Double-stranded DNA sequencing was performed according to the dideoxy chain-termination method (22) on an Applied Biosystems (Foster City, CA) 373A sequencer using the Taq DyeDeoxy Terminator cycle sequencing kit supplied by the same company. The type III/type IV and reverse primers (Qiagen) were used for sequencing.

Recombinant PLA₂ concentrations were determined according to the Bradford method, using a commercial kit (Bio-Rad, Richmond, CA) and commercial PLA₂ as a standard, or were calculated from the absorbance using an $\epsilon_{280}^{1\%}$ of 13.0 (23).

Recombinant antigens

AgS were expressed in *Escherichia coli* strain M15 bearing the pRep4 repressor plasmid (24), using the pDS56/RBSII, 6xHis expression plasmid (25) (kindly provided by Dr. D. Stüber, Hoffmann-La Roche, Basel, Switzerland). The expression of a synthetic gene encoding PLA₂ in this system and the isolation and characterization of recombinant wild-type Ag have been described in detail (26, 27).

The site-specific substitution of the catalytically essential and conserved histidine residue 34 with glutamine (H34Q) was introduced by PCR. A gene fragment carrying the H34Q mutation was amplified using the primer 5'-ACGCATGCTGTCGACCCAGGACA-3' together with the flanking T7 primer (Stratagene, La Jolla, CA) and the synthetic gene encoding the wild-type PLA₂ polypeptide in a pBluescript II SK vector as the target (26). PCR was performed in 100 μ l of sample containing the appropriate buffer, 200 μ M of each deoxynucleotide triphosphate, 0.5 μ M of each 5'- and 3'-primer, 10 ng of target DNA, and 1 U of Vent DNA polymerase (New England Biolabs, Schwalbach/Taunus, Germany). The samples were subjected to two cycles of amplification consisting of denaturing for 1 min at 95°C, annealing for 1 min at 45°C, and extending for 1 min at 72°C, followed by 25 cycles under identical conditions except that the annealing was done at 50°C. The PCR-product was purified by agarose gel electrophoresis and recovered with GeneClean (Bio 101 Inc., La Jolla, CA). This mutant gene fragment was digested with *Sph*I and *Hind*III and cloned into the same restriction sites of the wild-type PLA₂ expression construct (26). The presence of the mutation was confirmed by DNA sequencing, and constructs carrying the correct insert were selected for protein expression. The active site mutant PLA₂-H34Q was isolated, refolded, and purified as described for the wild-type counterpart (26, 27). Fresh resin and disposable plastic ware were used to exclude contamination of the mutant protein preparation with wild-type PLA₂.

Enzymatic assays

Enzymatic activities of the PLA₂ preparations were measured using the spectrophotometric assay with 2,3 bis-(hexanoylthio)propyl-1-phosphomethanol as substrate, following the previously described procedure (27) or using the pH-stat assay and 1,2 dimyristoyl-*sn*-glycero-3-phosphomethanol vesicles as a substrate (26).

Circular dichroism spectra

CD spectra were obtained using a J-720 spectropolarimeter (JASCO Instruments, Tokyo, Japan). The protein samples were dissolved in 10 mM sodium phosphate buffer, pH 7.0, at a concentration of 10 μ M. Spectra

⁵ Abbreviations used in this paper: PLA₂, phospholipase A₂ from bee venom; CD, circular dichroism; 5-HT, 5-hydroxytryptamine; RBL, rat basophilic leukemia (cells); hIgE-V_{NIP}, (4 hydroxy-3-nitrophenyl)acetyl-specific human IgE; HSA/NP, (4 hydroxy-3-nitrophenyl)acetyl coupled to human serum albumin; LDH, lactate dehydrogenase; hFcεR1 α , human high affinity IgE receptor α -chain.

(average of 12–16 scans) were collected over a range of 180 to 300 nm using a 0.2-mm path length cuvette.

Phospholipid vesicle binding

The binding affinity of PLA₂ and PLA₂-H34Q to phospholipid vesicles was measured by fluorescence resonance energy transfer between tryptophan residues of the enzyme and the probe *N*-dansyl-hexadecylphosphoethanolamine present at 2 mol % in co-vesicles of the nonhydrolyzable phospholipids 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol (20%)/1,2-ditetradecyl-*sn*-glycero-3-phosphocholine (80%) (100 μ M total phospholipid) in 10 mM Tris, 0.5 mM CaCl₂, pH 8.0 (28).

Antibody binding analysis

Relative binding affinities were determined by inhibition ELISA conducted as described (27). Briefly, purified recombinant Ag preparations were coated (0.1 μ M diluted in PBS, pH 8.0; 100 μ l/well) overnight at 4°C onto polystyrene microtiter plates (Maxisorb, Nunc, Denmark) followed by blocking of the remaining free sites with blocking buffer (PBS, pH 7.4, 1% casein hydrolysate (Oxoid Ltd., Basingstoke, U.K.), 5% (w/v) Tween 20). PLA₂-specific mAb at appropriate dilution was preincubated for 2 h at 37°C in a separate, untreated plate with serially diluted wild-type PLA₂ or PLA₂-H34Q. Thereafter this mixture was transferred to the Ag-coated plate and allowed to react for 2 h at 37°C. Bound Abs were detected with alkaline phosphatase-labeled secondary Ab followed by addition of 100 μ l of substrate and evaluated on the kinetic microtiter plate reader (27).

Release of 5-hydroxytryptamine (5-HT) and hexosaminidase from rat basophilic leukemia (RBL) cells

RBL-2H3 cells transfected with the gene for the human Fc ϵ receptor 1 α -chain (RBL-2H3 2/2/C) (29) were incubated in 24-well Costar (Cambridge, MA) plates ($2-4 \times 10^5$ cells/well) in a humidified environment of 5% CO₂ at 37°C for 24 h in a reaction volume of 0.4 ml with S-MEM (Life Technologies, Paisley, Scotland) containing 10% FCS, 5-HT (1 μ Ci/ml), 10^{-6} M dexamethasone in the presence or absence of serum from a bee venom-sensitive individual (diluted 1:10, estimated by ELISA to contain 400 ng/ml total IgE). Following this incubation period, cells were washed twice with 1 ml buffer A (120 mM NaCl, 5 mM KCl, 25 mM Pipes, 1 mM CaCl₂, 0.04 mM MgCl₂, 5.6 mM glucose, pH 7.4) and preincubated for a further period of 10 min at 37°C with the above buffer (0.5 ml). For the determination of 5-HT release, duplicate samples of cells were incubated with buffer A supplemented with 5'-(*N*-ethyl)carboxyamidoadenosine (100 μ M) and PLA₂ or PLA₂-H34Q (0.01–100 μ g/ml) at 37°C for 15 min. After cooling on ice, the supernatant was removed, and aliquots were removed for liquid scintillation counting. Release of hexosaminidase was determined as described (30). Percent release was calculated by the method of Siraganian (31). All data are normalized with regard to the value obtained for triggering with 1 μ g/ml (4 hydroxy-3-nitrophenyl)acetyl-specific human IgE (hIgE-V_{NP}) and 100 ng/ml (4 hydroxy-3-nitrophenyl)acetyl coupled to human serum albumin (HSA/NP) (29).

IL-4 production by RBL-2H3 (2/2/C) cells

RBL-2H3 (2/2/C) cells (29) were incubated in 6-cm petri dishes ($\sim 2.5 \times 10^6$ cells/dish) in a humidified environment of 5% CO₂ at 37°C for 24 h in a total reaction volume of 2.5 ml with S-MEM, FCS (10% v/v). Following incubation, cells were washed twice with 2.5 ml of buffer A (see above) and preincubated for a further period of 10 min at 37°C with the same buffer (2.5 ml) before challenge with buffer A supplemented with 50 μ g/ml PLA₂ or PLA₂-H34Q. After the indicated incubation period, plates were cooled on ice. The cell supernatant was removed, and the corresponding cell pellets were solubilized in 2.5 ml buffer A containing 0.1% Triton X-100. Aliquots of the cell pellet and supernatant fractions (1.5 ml) were precipitated with 4.5 ml of acetone (-20°C for 15 min), and the precipitate was collected by centrifugation ($14,000 \times g$ for 10 min). The pellets were dissolved in 100 μ l of SDS sample buffer and proteins were separated by 17% SDS-PAGE (32) and blotted onto nitrocellulose (33). IL-4 immunoreactive protein was detected with an anti-

serum raised in rabbits against the rat IL-4-derived peptide DSPLEIRIINTLNQV. A FAST search (34) of the database (SWISSPROT version 32.0) using this sequence revealed no significant sequence similarity with other known proteins. Rabbits were immunized s.c. with 0.5 mg/ml of peptide emulsified in CFA, followed by two injections in IFA (s.c., 0.5 mg/ml) at 15-day intervals. Four days after a final boost with peptide in PBS (i.v., 0.5 mg/ml), serum was collected and Ab titer was determined by ELISA. This antiserum recognizes at least three ~ 18 - to 21-kDa proteins secreted from CHO.K1 cells transfected with the gene encoding rat IL-4 (gift from Drs. A. N. Barclay and M. Puklavec, Medical Research Council (MRC), Oxford, U.K.) (35) on Western blots. An antiserum raised in rabbits against recombinant rat IL-4 (generous gift from Dr. M. Puklavec, MRC) recognized the same bands on immunoblots.

IL-4-associated biologic activity was measured by induction of MHC class II/la expression on rat B splenocytes. To obtain B splenocytes, rat spleen cells were washed twice in serum-free RPMI 1640 medium, followed by flash lysis with 5 mM of NH₄Cl (2 ml) at room temperature for 1 min. After addition of 20 ml of RPMI, the cell suspension was centrifuged for 5 min at 1400 rpm, cells were resuspended in 600 μ l of PBS/0.5% BSA, and 100 μ l of the following Abs to T cell markers were added: W3/25, W3/13, OX8, and OX-35 (all obtained from Serotec, Oxford, U.K.). The cells were incubated for 1 h at 4°C with gentle shaking, washed twice with 20 ml of RPMI and resuspended in 500 μ l of PBS/0.5% BSA. Dynabeads M450 coated with sheep anti-mouse IgG (Dyna 110.01, 500 μ l) were added, and cells were incubated for 1 h at 4°C with gentle shaking. Coated T cells were depleted using a magnetic particle concentrator, and the supernatant containing splenic B cells was collected. Following washing with RPMI, B cells were suspended in serum-free medium (Macrophage-SFM, Life Technologies) at a cell density of 7×10^5 cells/ml. Aliquots (300 μ l/well) of the cell suspension were plated out in a 24-well Costar plate, and 100 μ l of the supernatants released from RBL-2H3 2/2/C cells in response to incubation with PLA₂ and PLA₂-H34Q were added to each well. The spleen cells were incubated at 37°C in a humid atmosphere with 5% CO₂. After 24 h, cells were washed twice with serum-free RPMI and incubated for 1 h at 4°C with FITC-labeled Ab to rat class II/la molecules (OX6, Serotec U.K., 1:25 dilution in PBS/5% BSA). Before analysis by flow cytometry, cells were washed twice with PBS/1% BSA and resuspended in 500 μ l PBS/1% paraformaldehyde. An unrelated FITC-labeled Ab (anti-human IgE) was used to set the threshold for MHC class II positive staining.

Immunization of mice

CBA/J mice (6–8 wk of age) were purchased from Bomhite (Bomholtgard, Ry, Denmark) and were immunized by biweekly i.p. injections of low dose (LD: 0.1 μ g/injection) or high dose (HD: 10 μ g/injection) of PLA₂ or PLA₂-H34Q in 2 mg of Al(OH)₃. Sera were collected at day 35 after the first immunization and assayed for PLA₂-specific IgE and IgG Abs by ELISA. Assays were performed as described (18), except that standards for IgG (anti-PC-12-3) and IgE (anti-PC-56-1) (36) were included. Titers were determined by twofold serial dilutions of the sera starting with 1:50, and are defined as the serum dilution giving a signal equivalent to 0.4 ng/ml of IgE and IgG standard, respectively.

Results

IL-4 is known to play a central role in the induction and control of IgE Ab responses. However, the cellular origin of IL-4 as well as the mechanisms by which certain Ags stimulate the synthesis of this cytokine at a time when specific lymphocyte responses are not yet established are still unclear. Besides T lymphocytes, mast cells and basophils have been shown to produce considerable amounts of IL-4 following stimulation (37–39). The present study investigates the possibility that allergens might directly activate cells of mast cell/basophil lineage and induce IL-4 production by a nonimmunologic mechanism.

We used PLA₂, the main allergen of bee venom, to explore this possibility. Since endogenous phospholipase A₂ enzymes have been shown to cause mast cell activation

(21, 40), we hypothesized that PLA₂ might activate mast cells by virtue of its enzymatic activity. We therefore generated PLA₂-H34Q, a PLA₂ variant lacking enzymatic function, by substituting the catalytically essential and evolutionary conserved histidine residue 34 (19, 20) with glutamine, and compared its mast cell secretagogue activity to its enzymatically active wild-type counterpart.

Both PLA₂ and PLA₂-H34Q were isolated from the same *E. coli* high level expression system (26). As predicted by the proposed enzymatic mechanism (19), PLA₂-H34Q exhibited no detectable enzymatic activity ($<10^{-4}$ of wild-type-specific activity). However, PLA₂ and PLA₂-H34Q had comparable secondary structure as assessed by CD spectroscopy. Mutant and wild-type PLA₂ lack free thiols when analyzed with Ellmann's reagent and mass spectrometry (26), indicating that all five disulfide bonds have been formed. Fluorescence energy transfer experiments show that wild-type and mutant PLA₂ display tight and saturable binding to phospholipid vesicles (Fig. 1A). While this type of analysis will not yield an accurate dissociation constant because the affinity is so high, it is apparent that both PLA₂ and PLA₂-H34Q bind to phospholipid vesicles with nanomolar affinity, which is a hallmark of 14-kDa phospholipases A₂ (41). It is also apparent that the efficiency of energy transfer is comparable for both molecules, suggesting that position and orientation of the tryptophan residue involved in energy transfer is not significantly altered. Immunologic analysis shows that mutant and wild-type Ag exhibit the same affinity to a conformation-specific human mAb directed against the K25 epitope (27) (Fig. 1B). In addition, PLA₂ and PLA₂-H34Q provoke comparable type I skin reactions in bee venom allergic individuals (42). Thus, PLA₂-H34Q folds into a native-like conformation, and the lack of enzymatic function is not due to a disruption of the correct three-dimensional structure.

Degranulation of RBL cells

We first tested the ability of PLA₂ and PLA₂-H34Q to degranulate rat basophilic leukemia cells transfected with the gene encoding the α -chain of the human high affinity IgE receptor complex (hFc ϵ R1 α) (RBL-2H3 2/2/C clone) (29) in the presence or absence of sensitization with serum from an individual allergic to bee venom. RBL cells represent an accepted model system for the study of mucosal mast cell function (43). Following sensitization, both PLA₂ and PLA₂-H34Q induce mediator secretion, as monitored by the release of [³H]5-hydroxytryptamine (5-HT), from transfected cells with comparable efficiency, as demonstrated by the bell-shaped dose-response curve observed for both Ags (Fig. 2). This dose-response curve is characteristic of mast cell stimulation induced by receptor-bound IgE cross-linking. The comparable potency of PLA₂ and PLA₂-H34Q shows that both Ags are equally recognized by IgE Abs and provides additional confirmation that

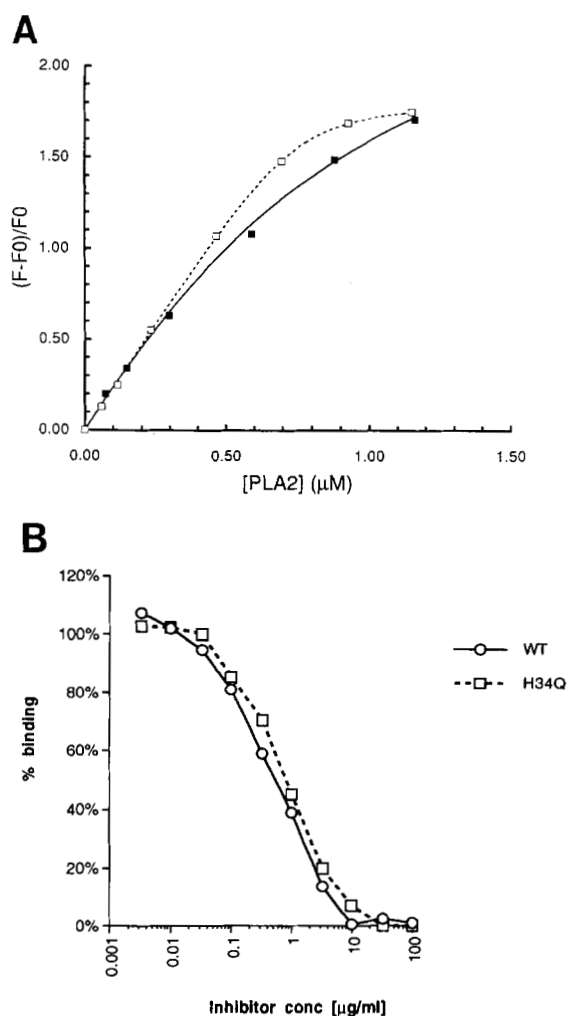


FIGURE 1. A, Binding of PLA₂ and PLA₂-H34Q to phospholipid vesicles. Binding affinity was estimated from the relative fluorescence emission enhancement due to energy transfer from tryptophan side-chains on PLA₂ (closed symbols) or PLA₂-H34Q (open symbols) to the dansyl fluorophore present at 2 mol % in nonhydrolyzable phospholipid vesicles as described in *Materials and Methods*. B, Binding of PLA₂ and PLA₂-H34Q to the conformation-dependent human mAb BVA1 specific for the K25 epitope (27). Relative binding affinities were determined by inhibition ELISA, in which serially diluted PLA₂ or PLA₂-H34Q was preincubated with BVA1 followed by transfer of the mixture to a wild-type Ag-coated plate. Following incubation, Ab binding was measured as described in *Materials and Methods*. The extent of binding is expressed as percent of reactivity in the absence of inhibitor, after subtracting background for each point.

PLA₂-H34Q displays no significant change in the tertiary conformation.

In contrast, when hFc ϵ R1 α transfectants are incubated with the same concentration of Ag in the absence of sensitizing serum, active PLA₂ causes degranulation (Fig. 2), and mediator secretion increases with Ag dose, whereas

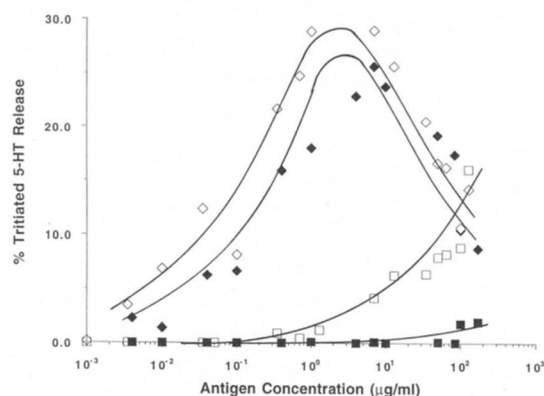


FIGURE 2. 5-HT release from RBL-2H3 2/2/C cells incubated with PLA2 and PLA2-H34Q. RBL-2H3 cells transfected with the gene for hFcεR1α (29) with (diamonds) or without (squares) sensitization with allergic human serum were challenged with varying amounts of PLA2 (open symbols) or PLA2-H34Q (closed symbols). 5-HT release was measured after a 15-min incubation at 37°C. Percent release was calculated by the method of Siraganian (31). Each symbol represents the mean of two or three tests carried out in duplicate. All data are normalized with regard to the value obtained for triggering with 1 µg/ml of hIgE-V_{NP} and 100 ng/ml of HSA/NP (29).

the inactive variant PLA₂-H34Q induces only marginal release at the highest Ag concentration. Release patterns similar to those observed for 5-HT were obtained for hexoseaminidase (data not shown). Thus, the enzymatic activity of PLA₂ seems to promote IgE-independent mast cell activation.

Induction of IL-4 synthesis in RBL cells

Previous reports demonstrated *de novo* protein synthesis and secretion of cytokines, including IL-4, by mast cells in response to FcεR1 cross-linking (37, 38). Since the enzymatic activity of PLA₂ appears to promote IgE independent mast cell activation, we monitored, over a period of 6 h, the protein content in pellets and supernatants of RBL cells that had not been sensitized with IgE, before and after addition of PLA₂ and PLA₂-H34Q to the medium bathing the transfected cell line. During this period, the protein concentration in the supernatants of cells exposed to PLA₂ rises from ~30 µg/ml to ~390 µg/ml. The corresponding increase in unstimulated cells or those exposed to PLA₂-H34Q is less than 50 µg/ml.

The presence of IL-4 in the cell pellet and supernatant fractions of these samples was demonstrated by Western blot analysis using an antiserum raised in rabbits against a rat IL-4-derived peptide or a polyclonal rabbit anti-IL-4 Ab (generous gift of Dr. M. Puklavec, MRC Oxford). The immunoblot (Fig. 3) shows that PLA₂ stimulates a time-dependent expression and release of IL-4 from RBL 2H3 2/2/C cells. Several polypeptide bands of ~18 to 22 kDa

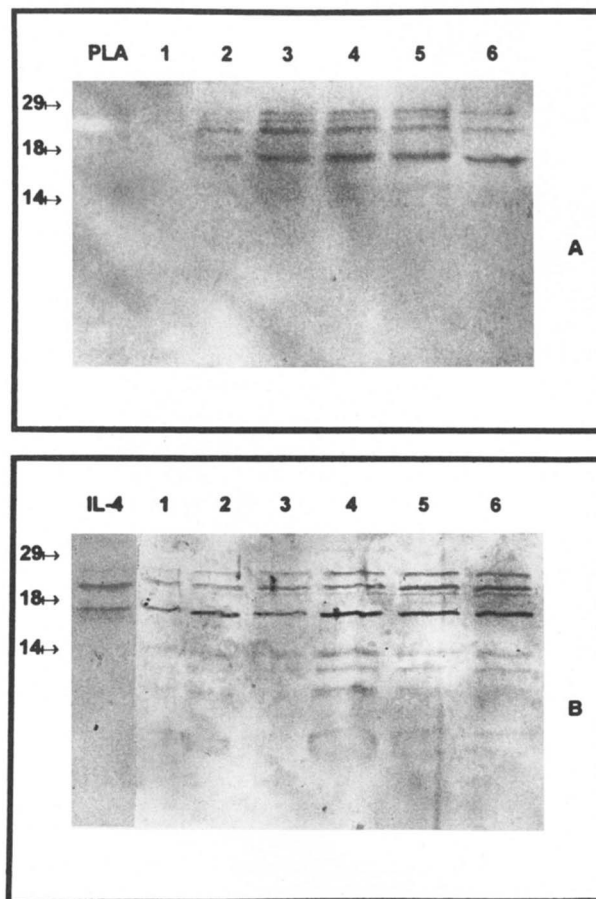


FIGURE 3. Kinetics of IL-4 induction in RBL-2H3 (2/2/C) (29) cell pellets and release following exposure to PLA2. RBL-2H3 (2/2/C) cells (29) were challenged with 50 µg/ml of PLA2 or PLA2-H34Q in the absence of sensitizing IgE. After the indicated incubation period, proteins present in culture supernatants or cell pellets were resolved by SDS-PAGE and analyzed by immunoblotting with Ab to IL-4-derived peptide. Anti-IL-4 Ab was detected by horseradish peroxidase-labeled anti-rabbit Ab. The first lane in panel A represents the control for PLA2 in buffer A employed for cell challenge, and in panel B the first lane corresponds to the supernatant of CHO.K1 cells transfected with the gene encoding rat IL-4 (35). Lanes 1–6 represent cell supernatants (A) and pellets (B) before and 15, 60, 120, 240, and 360 min after challenge with PLA2. No IL-4 immunoreactivity was detected in cell supernatants, and no increase of the basal cellular levels of IL-4 was observed following challenge with PLA2-H34Q (not shown). Comparable results were obtained using an Ab raised against recombinant rat IL-4 (generous gift from Dr. M. Puklavec, MRC).

are detected in cell pellets; they probably represent the different glycosylated forms of IL-4 reported by others (35) and pro-IL-4. Within 15 min after addition of PLA₂, the transfectants secrete detectable quantities of IL-4 into the cellfree fraction. Additional bands of higher mobility appear within an hour in the cell pellet, but not in the supernatant fraction, of PLA₂-stimulated cells. These are

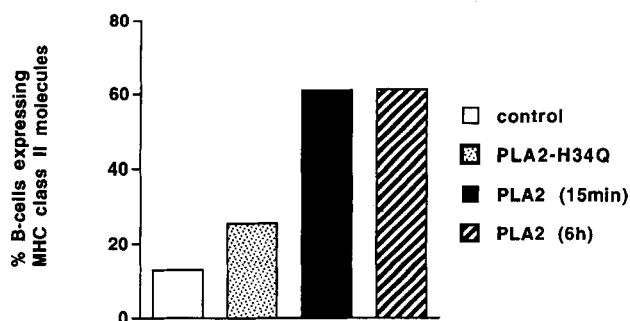


FIGURE 4. Up-regulation of MHC class II expression on rat B splenocytes by RBL-2H3 supernatants. Splenic B cells were isolated through immunomagnetic depletion of splenic T cells, as described in *Materials and Methods*. After isolation, B splenocytes were incubated with supernatants of RBL cells that have been treated with buffer alone (control) or challenged with 50 μ g/ml of PLA₂-H34Q (15 min) or PLA₂ for 15 min and 6 h, respectively. Following incubation, B splenocytes were stained with FITC-labeled Ab to rat class II molecules and analyzed by flow cytometry. The plot shows the % MHC class II-positive B cells following treatment with RBL supernatants, as described in *Materials and Methods*.

most likely incomplete translation products and point to a stimulation of de novo protein synthesis, which peaks between 2 and 4 h. Cells incubated with PLA₂-H34Q under identical conditions do not respond with mediator secretion, and no increase in cellular IL-4 is observed during the 6-h period (data not shown). These observations indicate that PLA₂ fuels rapid secretion of IL-4 and transient increase in IL-4 synthesis, a finding that had been made earlier when mast cells and basophils responded to an immunologic stimulus (37–39).

To obtain independent evidence for IL-4 production by RBL 2H3 cells following nonimmunologic stimulation by PLA₂, we measured MHC class II expression on primary rat B splenocytes in response to supernatants of RBL 2H3 cells. Up-regulation of class II Ag expression on B splenocytes is a characteristic action of IL-4 (35). Figure 4 shows that only mast cells exposed to PLA₂ secreted component(s) that enhanced expression of MHC class II/Ia Ags, whereas class II/Ia expression induced by supernatants of RBL 2H3 cells challenged with PLA₂-H34Q increased only marginally. Moreover, a polyclonal rabbit anti-IL-4 Ab (generous gift of Dr. M. Puklavec, MRC) blocked MHC class II up-regulation (data not shown), providing compelling evidence that rat mast cells secrete IL-4 in response to PLA₂ in the absence of IgE sensitization.

Induction of IgE Ab response in vivo

To test whether this enzyme-induced mediator secretion by PLA₂ might be of significance for Ag-specific immune responses in vivo, we compared isotype-specific Ab responses elicited by PLA₂ and PLA₂-H34Q following immunization of mice. Previous investigations employing

purified natural PLA₂ have shown that CBA/J mice are high responders with respect to both IgG and IgE Ab production. These studies further established that the prevalence of the isotype is critically dependent on the Ag dose (18): repeated injections of low doses of Ag (0.1 μ g/injection) induced high titers of PLA₂-specific IgE, whereas immunizations with high Ag doses (10 μ g/injection) elicited high levels of Ag-specific IgG. We followed the immunization schemes established by this model to assess the influence of the enzymatic activity on isotype selection in the Ab response against PLA₂. The results of a typical experiment in which identical sets of high Ag dose and low dose immunization procedures were conducted in CBA/J mice using PLA₂ and PLA₂-H34Q as Ags are shown in Figure 5. The outcome demonstrates that repeated injections with low doses of wild-type Ag induces high levels of PLA₂-specific IgE, while immunization with PLA₂-H34Q under identical conditions yields no detectable PLA₂-specific IgE synthesis. In the latter case, PLA₂-specific IgG levels were significantly lower compared with those elicited by wild-type Ag, but detectable IgG1 and IgG_{2a} responses were found in every animal.

In contrast, when high doses of Ag were used for immunization, the isotype pattern and specific Ab levels were found to be similar for both PLA₂ and PLA₂-H34Q. These results indicate that PLA₂ and PLA₂-H34Q are similarly immunogenic at high dose; at low dose, however, enzymatically active PLA₂ induces high titer IgE Ab responses under conditions in which PLA₂-H34Q is only weakly immunogenic.

To assess whether the differences observed reflect merely a shift in the dose-response profile between PLA₂ and PLA₂-H34Q, mice were immunized with intermediate doses of mutant Ag. Although PLA₂-H34Q exhibits a gradual increase in immunogenicity, as shown by the dose-dependent rise in specific IgE and IgG levels, a high titer IgE response comparable to the wild-type Ag is not induced by any dose of PLA₂-H34Q. Specific IgE levels reached a maximum in mice immunized with 1 μ g/injection, and titers were equal to those observed in mice immunized with 10 μ g/injection of Ag. Thus, the high titer IgE Ab response of PLA₂ appears to be intrinsically associated with its enzymatic activity and does not occur with any dose of its enzymatically inactive counterpart.

Discussion

A central issue in the development of IgE-mediated allergies is the characteristics of an allergen that promotes IL-4 synthesis at a stage where specific lymphocyte responses are not yet established and thus drives Ab responses towards a predominant IgE isotype. Regarding the cellular source of IL-4 at the onset of immunity, a recently identified CD4⁺NK1.1⁺ rare T cell population, which promptly produces IL-4 following challenge with anti-CD3 or superantigen, has been proposed to play a role in

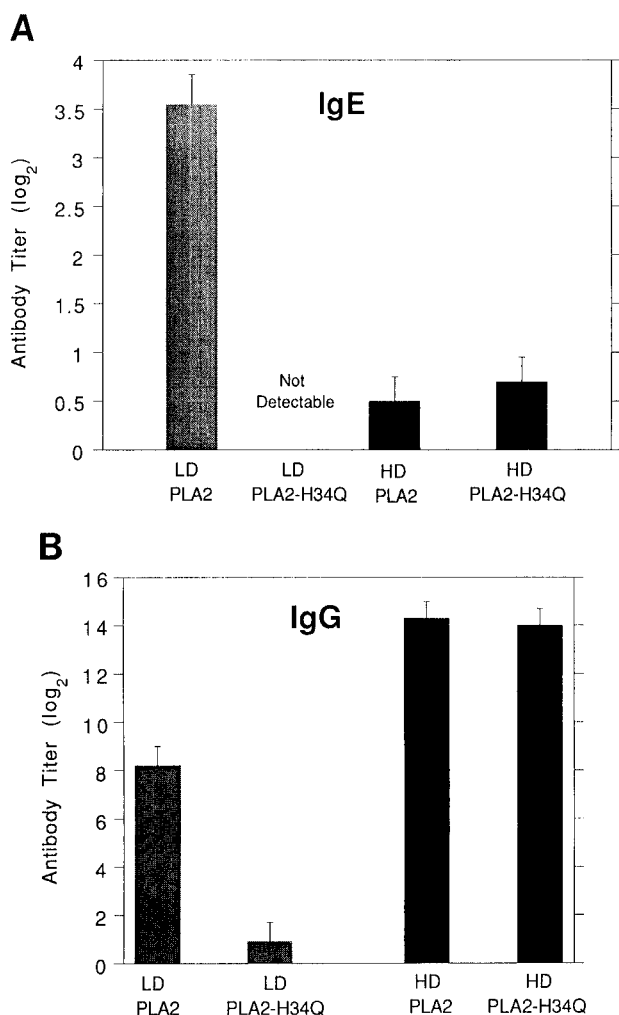


FIGURE 5. Isotype pattern of PLA₂-specific Abs induced by PLA₂ and PLA₂-H34Q. CBA/J mice were immunized with low dose (LD, 0.1 μ g/injection) or high dose (HD, 10 μ g/injection) of Ag. Sera were collected at day 35 after the first immunization and assayed for PLA₂-specific IgE (A) and IgG (B) Abs by ELISA. At this time the isotype pattern is established and Ab titers do not change thereafter. Ab titers were determined by ELISA and were normalized using IgE and IgG reference standards as described in *Materials and Methods*. Each bar represents the arithmetic mean (+SE) of a group of four animals, and data are expressed as log₂ of Ab titers. Comparable isotype patterns were observed in three sets of immunizations.

IL-4-dominated immune responses; it has been speculated that allergens or nematodes might have associated superantigens that enable them to induce IL-4 production during the priming of antigen-specific T cells (44). Alternatively, the source of IL-4 at the onset of immunity might be provided by mast cells or basophils, and it has been speculated that the interaction of helminths or allergens with these cells may induce IL-4 release (8, 9). The difficulty with the later possibility is that the only known physiologic stimulus inducing IL-4 production by these

cells is cross-linking of Fc ϵ R1 (37–39), and this would normally occur only after specific IgE Ab responses are already established.

In the present study we investigated the possibility that an Ab-independent interaction of an allergen such as PLA₂ with mast cells or basophils might also stimulate IL-4 production. In the light of the suggested role of endogenous phospholipase A₂ enzymes in mast cell activation (21, 40), we hypothesized a direct enzymatic action mode of the PLA₂ allergen in inducing mast cell activation. Thus, we measured mast cell stimulation by PLA₂ and PLA₂-H34Q, a mutant that is structurally and functionally indistinguishable from wild-type Ag except that it lacks enzymatic activity. We have shown that in rodent mast cells PLA₂ triggers IgE-independent mediators release (Fig. 2) and induces de novo synthesis and secretion of IL-4 (Figs. 3 and 4), demonstrating that allergens can stimulate IL-4 production by cells of mast cell lineage by a nonimmunologic mechanism. The finding that these activities cannot be observed with the enzymatically inactive variant further suggests that the stimulatory effect is due to the enzymatic function of PLA₂.

The simplest interpretation of all these findings would be that PLA₂ activates mast cells in a direct enzyme-mediated process by hydrolyzing membrane-bound phospholipids, and that the products of phospholipid hydrolysis might trigger mast cell degranulation. In support of this view, endogenous type II phospholipase A₂ has been associated with mast cell degranulation (21, 40). Thus the PLA₂ allergen, by virtue of its lipolytic activity, may simply mimic the metabolic signals normally generated by the cellular phospholipase A₂ and bypass the cellular control of mast cell activation.

It might be argued that mediator release from RBL cells could result from a loss of cell integrity due to membrane phospholipid degradation by PLA₂. We have addressed this issue by measuring release of lactate-dehydrogenase (LDH), a cellular enzyme released upon cell disruption into culture supernatants following incubation with 50 μ g/ml of PLA₂. We found that, during a 2-h incubation period under conditions in which mediator release and IL-4 secretion occurs, <8% of the LDH was released from RBL cells. In contrast to that, incubation of RBL cells under identical conditions with 50 μ g/ml of bee venom resulted in 100% LDH release and in a visible loss of cell integrity. Therefore, we believe that the cytotoxic effects reported for (commercially available) PLA₂ by some investigators in the past are most likely due to contamination with other bee venom components such as mellitin, a known cytolytic peptide which is abundant in bee venom.

Another hypothesis would be that the PLA₂ allergen activates mast cells by binding to a putative receptor. Recent studies have identified a high affinity receptor for type I phospholipase A₂ in various tissues (45, 46), and a brain receptor for potent neurotoxins has been shown to bind several phospholipase A₂ enzymes from various species

with high affinity (47, 48). Interestingly, the bee venom enzyme has also been reported to bind to a neurotoxin receptor in rabbit skeletal muscle with nanomolar affinity (49). Since the only conserved structural feature of these diverse phospholipase A₂ enzymes lies in the catalytic site, it is conceivable that PLA₂ might bind to these receptors via its active site. It is presently unknown, however, whether these receptors are also present on mast cells.

The observed link between enzymatic activity and IgE-independent mast cell activation by PLA₂ implies that only catalytically active Ag should efficiently initiate IgE Ab synthesis in vivo, a prediction we confirm using the experimental murine system.

In Figure 5 we show a preferential induction of IgE Abs as a result of repeated low dose immunization with enzymatically active, but not inactive, PLA₂. It should be noted that under low dose immunization conditions, PLA₂-H34Q is considerably less immunogenic than its wild-type counterpart, whereas no difference in IgG or IgE responses is apparent when animals are immunized with high doses. Thus, the enzymatic activity of PLA₂ is not only associated with predominant IgE responses but also leads to an increased immunogenicity at low dose. It could be argued that the difference in immunogenicity might be due to the disruption of a dominant T helper cell epitope by the H34Q mutation. When the proliferative response of lymph node cells from mice immunized with 0.1 µg/injection of PLA₂ was measured in vitro after stimulation with 10 µg/ml PLA₂, PLA₂-H34Q, or control buffer, wild-type and mutant Ag incorporated 32,933 ± 1,616 and 32,890 ± 1,821 cpm, respectively, compared with 543 ± 312 cpm in the buffer control.

Thus, we find no evidence of an immunodominant T helper cell epitope spanning the region of H34, but since the T cell epitopes on PLA₂ in this or any other mouse strain are at present unknown, we cannot rule out the possibility that an altered T helper epitope can contribute to the different immunogenicities observed in vivo. In the light of the demonstration of enzyme-mediated activation mast cells, however, we favor a direct enzymatic action mechanism in which PLA₂ induces IL-4 synthesis by mast cells during the priming of specific lymphocyte responses, which finally results in a predominant IgE Ab response.

The present investigation has focused on the biologic action of the Ag on rodent mast cells and IL-4 secretion; the release of additional mediators from these and possibly other cell types in response to Ag will be considered in further investigations. It is interesting to note in this context that many airborne and food-associated sources of allergens comprise lectins and enzymes such as phospholipases and proteases (2, 50, 51). The latter are also released by invading parasites, bacteria, and viruses. We and others have shown that proteases, including those of house dust mite, fungal, bacterial, or schistosomal origin, can degranulate mast cells by a nonimmunologic mechanism (2, 50, 51). This suggests that a common mechanism may de-

termine the isotype response to allergens or parasite Ags. It is interesting to note in this context that dose-dependent isotype responses comparable to those elicited in response to active PLA₂ were observed when the infection by the nematode *Nippostrongylus brasiliensis* was studied in rats (52). Confirmation of this hypothesis should have important consequences for the design of vaccination schedules to prevent IgE-mediated allergies and parasitic infestations.

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