

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

T-cell and antibody responses to phospholipase A₂ from different species show distinct cross-reactivity patterns

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Abstract

Background: Secreted phospholipases A₂ (sPLA₂) represent antigens to which humans may be rarely or frequently exposed. Thus, the investigation of humoral and cellular immune responses to sPLA₂s from different species can provide a suitable model in the study of antibody and T-cell cross-reactivity.

Methods: Specific IgE, IgG₁, IgG₄, and IgA antibodies were analyzed by ELISA against sPLA₂s from pancreas of *Bos taurus* (BT), *Apis mellifera* (AM) bee venom, *Daboia russellii* (DR) and *Naja mossambica* (NM) snake venoms, and human group III (hGIII) sPLA₂ using sera of nonallergic beekeepers, AM-allergic patients, and healthy controls. T-cell cross-reactivity was investigated in PBMC, and T-cell clones (TCC) are generated against AM sPLA₂.

Results: Hyperimmune and allergic individuals showed high levels of sPLA₂-specific IgG₄ and significant IgG₄ cross-reactivity between BT, DR, and NM sPLA₂s. Furthermore, IgE, IgA, and IgG₁ cross-reactivities against BT, DR, and NM sPLA₂s were also detectable in the range of 22.2–44.8%. Allergic patients showed significant T-cell proliferative response to NM sPLA₂ together with increased IFN-γ and IL-13 production even though they had never been exposed to cobra venom. Although nonallergic healthy controls show no cross-reactivity at T-cell level, they did have low levels of IgG₄ and IgA against BT, DR, and NM sPLA₂s. Human TCC spanning three major T-cell epitopes of AM sPLA₂ showed minor proliferative response to NM and hGIII sPLA₂s.

Conclusions: This study shows that T cells and antibodies may show cross-reactivity between different species without being naturally exposed to sPLA₂s.

Humans are exposed to a number of secreted phospholipases A₂ (sPLA₂s) through a variety of sources in their life. sPLA₂s constitute a large family of structurally related enzymes that are widely present in nature. sPLA₂s were first identified in snake (group I and II) and bee venoms (BV) (group III), later in mammalian pancreatic fluid (group IB) (1, 2), and finally in various human tissues by cloning several isoforms including the human group III (hGIII) sPLA₂ that is homologous to BV (2, 3).

Biological functions and pharmacological effects of snake venom and mammalian sPLA₂s are highly diversified. They play a role in neurotoxicity, myotoxicity, lipid digestion, cell proliferation, host defense, and inflammatory diseases (4–6).

Secreted phospholipases A₂ from different species may also have antigenic or allergenic properties. For instance, BV sPLA₂ is the major allergen responsible for sensitization after bee sting (7). In addition, it represents a suitable model for studying the immunologic cross-reactivity to other sPLA₂s, including humans who have never been exposed to venom sPLA₂s. Several studies reported varying degrees of cross-reactivity between venoms from snake or bees (8, 9). Cross-reactivity among animal mammalian proteins such as milk and meat allergens has also been reported to occur when they share common epitopes (10). Consequently, cross-reactivity of IgE and other antibody isotypes to homologous sPLA₂s from different species is an interesting topic to address. Addi-

tionally, to understand the nature of allergy in detail, it is important to examine the cross-reactivity of sPLA₂s originated from phylogenetically diverse species in individuals who are allergic or hyperimmune to bee venom.

Therefore, we studied T-cell activation, cytokine production, and specific antibody response to sPLA₂s from different species including hGIII, pancreatic sPLA₂ from *Bos taurus* (BT), and sPLA₂s from *Apis mellifera* (AM), *Daboia russellii* (DR), and *Naja mossambica* (NM) venoms.

Methods

Study population

Serum samples from eight BV-allergic patients, four beekeepers, and eight healthy individuals were studied for the analysis of specific IgE, IgG₁, IgG₄, and IgA antibodies against five different sPLA₂s. As beekeepers were hyperimmune subjects with very high PLA₂-specific IgG, particularly IgG₄ levels owing to repeated bee stings, we wanted to see whether this population had specific antibodies against other sPLA₂s in the sera. In addition, Peripheral blood mononuclear cells (PBMCs) of five allergic patients and five healthy controls were examined for T-cell proliferation and cytokine responses. Patients who had a history of systemic reactions after bee sting showed positive skin test responses (ALK, Horsholm, Denmark) and had more than 2 kU/l specific IgE (CAP immunoassay; Pharmacia, Uppsala, Sweden).

Database mining and amino acid alignment

Multiple alignments performed using various sPLA₂s are now shown in Fig. 1. However, there exist at least two isoforms for PLA₂s, and unfortunately, genomic DNA sequences for all species, especially for NM (*Naja mossambica*) and DR, is not available. Additionally, it is almost impossible to make perfect alignment between GI/II and GIII PLA₂s because of high diversity of protein sequence apart from active sides and Ca²⁺-binding loop regions of all PLA₂s. Therefore, we have separated our alignment into three subgroups: alignment for GIII, GI/II, and active sites of all PLA₂s. The amino acid sequences of sPLA₂s including AM bee venom, hGIII, BT, NM, and DR are extracted from NCBI database and UniProt data bank and aligned using the default options of CLUSTALW2.1 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The alignments are shaded using Boxshade server (http://www.ch.embnet.org/software/BOX_form.html). Identity and similarity matrix for respective alignment is calculated using the program 'GeneDoc' (11). The accession numbers for all protein sequences are indicated in legend to Fig. 1.

Recombinant production of hGIII sPLA₂ protein and preparation of polyclonal antibodies against hGIII protein

Human group III sPLA₂ domain (3) was produced in *Drosophila* S2 cells according to the protocol that is described previously for human sPLA₂ group IID (12) and mouse

group III sPLA₂ (13). Protein was purified to homogeneity by successive purification on anion-exchange and reverse-phase HPLC columns. Mass spectrometry analysis by MALDI-TOF, N-terminal sequencing, and deglycosylation experiments performed using N-glycopeptidase F showed that the recombinant protein is properly folded. The recombinant protein is N-glycosylated at two positions (data not shown). Each N-linked glycan was found to have a molecular mass of 1038 Da, which is comparable with glycans found in other proteins expressed in S2 cells (14). Similar N-glycan moieties were also observed when mGIIC and mGIII sPLA₂ domain were expressed in *Drosophila* S2 cells (13). However, further analyses of the molecular structure of N-glycan moieties are not performed. Rabbit polyclonal antiserum, which is raised against hGIII sPLA₂, was prepared by the injection of purified recombinant protein to rabbit. Polyclonal hGIII antiserum was used at a working dilution of 1/2000. In these conditions, antiserum was found to be specific for hGIII and show no cross-reactivity up to 50 ng of purified bvsPLA₂ (15).

Gel electrophoresis and Western blot using different human sera from allergic patients, beekeepers, and anti-bvPLA₂ IgG₄mAb

Antigen extracts with an equal volume of sample buffer containing no reducing agent were heated at 80–90°C for 5 min and then centrifuged for 1 min at 17949 g. AM, DR, and BT sPLA₂s at concentration of 5 mg/ml and NM sPLA₂ at 1 mg/ml were separated in NuPAGE Bis-Tris gel (Invitrogen, Basel, Switzerland) at 190 V for 1 h (BioRad, Richmond, CA, USA) (16).

Different sera were tested by Western blot for their cross-reactivity against 50 ng of purified hGIII sPLA₂. Sera from allergic patients and beekeepers were assayed at a working dilution of 1/500. Western blots for human antisera were revealed using anti-human IgG₄ secondary antibodies from Becton Dickinson, Allschwil, Switzerland (1/5000). Western blot analyses were performed according to the manufacturer's recommendations (NETG buffer) (15).

Measurement of sPLA₂-specific antibodies

Specific IgE, IgG₁, IgG₄, and IgA antibodies to AM, BT, DR and NM sPLA₂s were analyzed by ELISA as previously described (17, 18). Human monoclonal antibody (mAb) of IgG₄ isotype specific for Api m1 bee venom sPLA₂ (BVA2) was used as standard for anti-PLA₂IgG₄ (19). Plates were coated with 10 µg/ml sPLA₂s in PBS overnight at 4°C. They were then incubated with sera for 2 h at room temperature. Serum samples were 1 : 2 diluted and titrated 1 : 2 for eight steps, which ended at 1 : 256 dilutions. Same dilutions for both IgE and IgG₄ were used. Arbitrary units were determined by fitting the curve to arbitrary standard. Anti-human IgE mAb (clone TN 142, 1 µg/ml) (BD Pharmingen, Allschwil, Switzerland), anti-human IgG₄ clone RJ4 MoAb (Bionostics Inc., Devens, MA, USA), and anti-human IgA_{1/2} MoAb (Pharmingen) were used to develop anti-sPLA₂ Abs (20). We performed inhibition ELISAs to further investigate

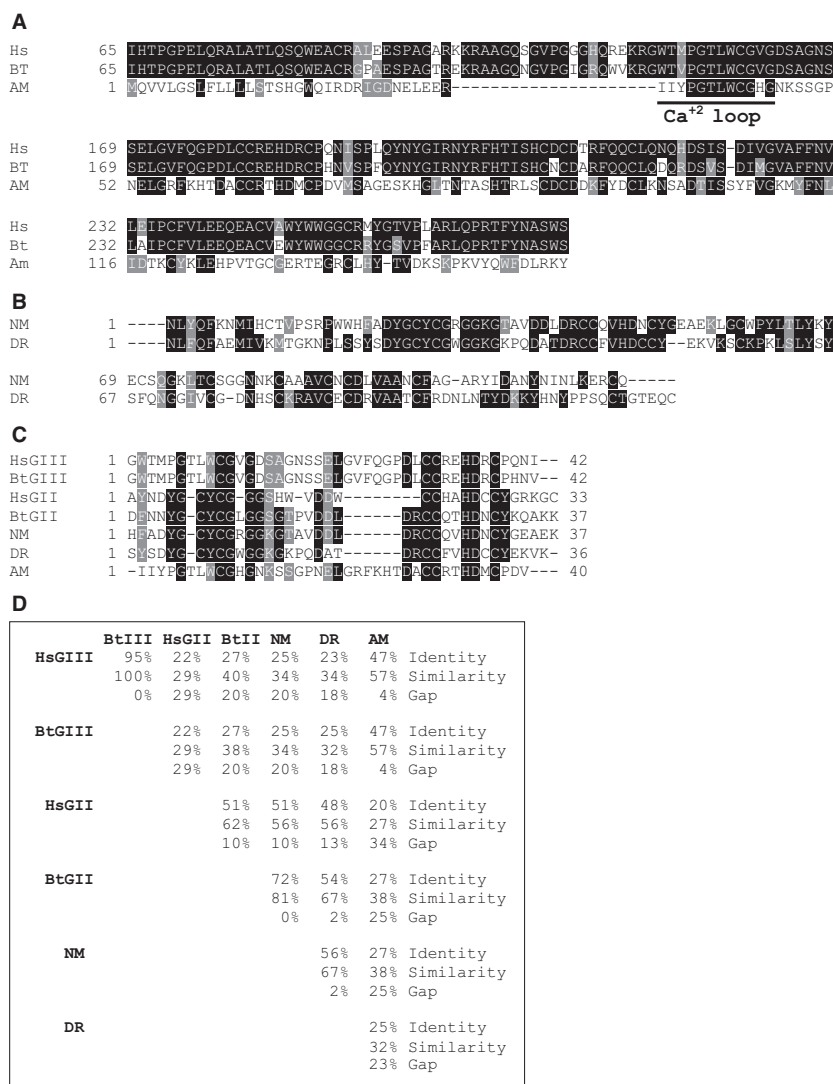


Figure 1 Multiple alignments of GIII and GI/II PLA₂s and active sites of secreted phospholipases A₂ (sPLA₂s). (A) Amino acid alignment of GIII sPLA₂. Hs Human (*Homo sapiens*) AF220490, Bt (*Bos taurus*) NM_001080910, Am (*Apis mellifera*) NP_001011614. (B) Amino acid alignment of GI/II sPLA₂s, Nm (*Naja mossambica*)

P00603, Dr (*Daboia russellii*) P81458. (C) Amino acid alignment of active sites of all sPLA₂s is shown. (D) Identity and similarity matrix of the active sites of all sPLA₂s is presented. Please see Methods for the details of the analyses.

antibody cross-reactivity between sPLA₂s. Sera from two beekeepers and two allergic individuals are pooled and checked for inhibition of binding to solid-phase-coated sPLA₂s by soluble sPLA₂s (21).

Quantification of cytokines

Solid-phase sandwich ELISAs for IFN- γ , IL-4, IL-10, and IL-13 have been previously described (22). Briefly, 96-well microtiter plates (Maxisorb, Roskilde, Denmark) were coated with mAb 43-11 to human IFN- γ . Sensitivity was 30 pg/ml. To detect IL-13, mAb JES 10-5A2 (Pharmingen) was used for coating. Detection limit was 50 pg/ml. IL-10 was determined using mAb JES3 19F1 for coating. Sensitivity

was 50 pg/ml. IL-4 was measured by coating with mAb 8F12 and biotin-conjugated mAb 3H4. Detection limit was < 100 pg/ml.

T-cell cultures

Peripheral blood mononuclear cells were isolated by Ficoll (Biochrom AG, Berlin, Germany). Cells (10⁶/ml) were stimulated with different doses (0.3, 0.06, and 0.012 μ M) of sPLA₂s or left unstimulated. After 5 days, supernatants were harvested for the measurement of cytokines and stored at -20°C until the analyses were performed.

Specific proliferative responses were determined in triplicate and pulsed with 1 μ Ci/well [³H] thymidine on day 5 for

8 h. Incorporated radioactivity was measured in Pharmacia Wallac beta plate reader (Turku, Finland) (20). Three T-cell clones (TCC) generated against BV sPLA₂ were tested in response to different sPLA₂s from PBMCs of allergic and healthy individuals. TCC specific to Api m1 and T-cell line specific to Api m2 were derived from hyper-immunized healthy individual as described (23). Clone 5.5E7 recognizes an epitope spanning amino acids 94–111, 5.6B8 recognizes an epitope spanning amino acids 81–98, and 5.6H2 recognizes an epitope spanning amino acids 113–124 of Api m1. In addition, Api m1-nonspecific clones 5.1F1 and 7.3B4 were used as controls. Epstein–Barr virus-transfected autologous B cells were used as APC (23). Experiments were performed in dose and time kinetics. Optimum proliferation and cytokine production at 0.6 μ M antigen doses are shown.

Statistical analysis

Data were compared by variance analysis. If homogeneity of variances is not available, then comparison of groups was analyzed by Kruskal–Wallis test and Z-test. To verify homogeneity of variances, Levene's test was used. Student's *t*-test was used for the comparison of two different groups.

Results

Amino acid sequence alignment, identity and similarity matrix between venom and mammalian sPLA₂s

Multiple alignments for the amino acid sequences of AM, hGIII, DR, NM, and BT sPLA₂s are shown in Fig. 1(A–C). We have found a relatively high amino acid sequence identity between AM, HsGIII, and BT. The identity and similarity of the corresponding amino acid region of GIII PLA₂s reach up to 88% and 94% between *Homo sapiens* (HS) and BT, and 31% and 48% between HS and AM, respectively. Furthermore, the identity and similarity of the corresponding amino acid region of GI/II PLA₂s are 41% and 48% between NM and DR, respectively. Sequence identities of the active sites of all sPLA₂s between HS, AM, DR, NM, and BT were higher (Fig. 1D). Protein bands of AM, NM, DR, BT, and hGIII sPLA₂s after SDS–PAGE analysis are shown in Fig. 2A. Apparent molecular masses of sPLA₂s are approximately as follows: AM, 16; NM, 14; DR, 10; and BT, 13 kDa. Only one major protein band was observed in the Western blot analysis of NM, DR, and BT sPLA₂s. Western blot for recombinant hGIII sPLA₂ (mW, 20 kDa) generated using rabbit antiserum is shown in Fig. 2B.

Specific IgE, IgG₄, IgG₁, and IgA and their cross-reactivities against different sPLA₂s

There were significant differences between AM-specific IgE, IgG₄, IgG₁, and IgA levels of controls and allergic patients/beekeepers ($P < 0.01$, $P < 0.01$). sPLA₂-specific IgA against NM was also significantly different between controls and allergic patients/beekeepers ($P < 0.05$). There were no detectable specific antibodies to hGIII sPLA₂ in controls, allergic

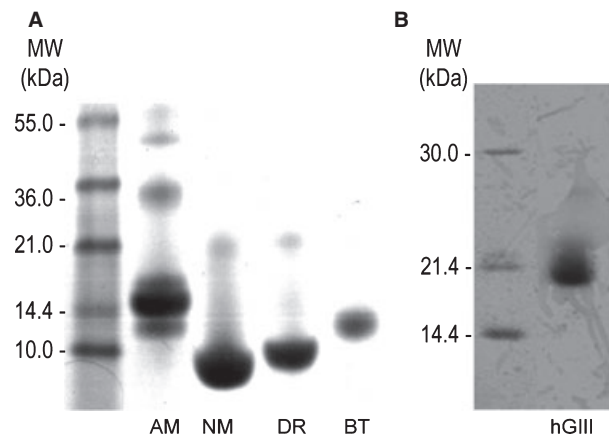


Figure 2 (A) Western blot analysis of AM, NM, DR, and BT secreted phospholipases A₂ (sPLA₂s). The molecular weights of sPLA₂s are approximately as follows: AM, 16; NM, 14; DR, 10; and BT, 13 kDa in lanes 2, 3, 4, and 5, respectively (lane 1 is the marker). Only one major protein band was visualized in NM and DR snake venoms and the pancreas of BT sPLA₂s. (B) Western blot of recombinant human group III sPLA₂ (mW, 20 kDa) with rabbit anti-serum. AM, *Apis mellifera*; BT, *Bos taurus*; DR, *Daboia russellii*; NM, *Naja mossambica*.

patients, and beekeepers. We have found detectable sPLA₂-specific IgG₄ Abs against BT, DR, and NM in sera of hyper-immune and allergic individuals (Fig. 3).

A significant IgG₄ cross-reactivity was observed against BT, DR, and NM sPLA₂s (100%, 100%, and 78% of studied serum, respectively). Specific IgE, IgG₁, and IgA cross-reactivities against BT, DR, and NM sPLA₂s were also detectable in sera of hyperimmune and allergic patients, ranging between 22.2% and 44.8%. IgE cross-reactivities against BT and DR were found in 3/12 and 2/12 subjects with AM-specific IgE, respectively. IgG₄ antibodies against BT, DR, and NM were observed in 9/16, 9/16, and 7/16 subjects having specific IgG₄ to AM, respectively. IgG₁ cross-reactivities against BT and DR were positive in 7/15 and 9/15 subjects with AM-specific IgG₁, respectively. Finally, IgA cross-reactivities against BT, DR, and NM were positive in 6/13, 4/13, and 4/13 individuals with AM-specific IgA, respectively.

Each sPLA₂ inhibited antibody binding to the same antigen when used in a soluble form. There was partial inhibition between different sPLA₂s in each experiment. The inhibitory capacity of other sPLA₂s against AM sPLA₂ was relatively weak. BT sPLA₂ showed very significant cross-reactivity and inhibition of Ab binding to BT sPLA₂, as it was observed by all other sPLA₂s (Fig. 4).

T-cell response to different sPLA₂s

Increased IFN- γ production was significantly induced by NM sPLA₂ in allergic patients. This was in correlation with their enhanced T-cell proliferation ($P = 0.004$). However, there was a significant difference between controls and allergic

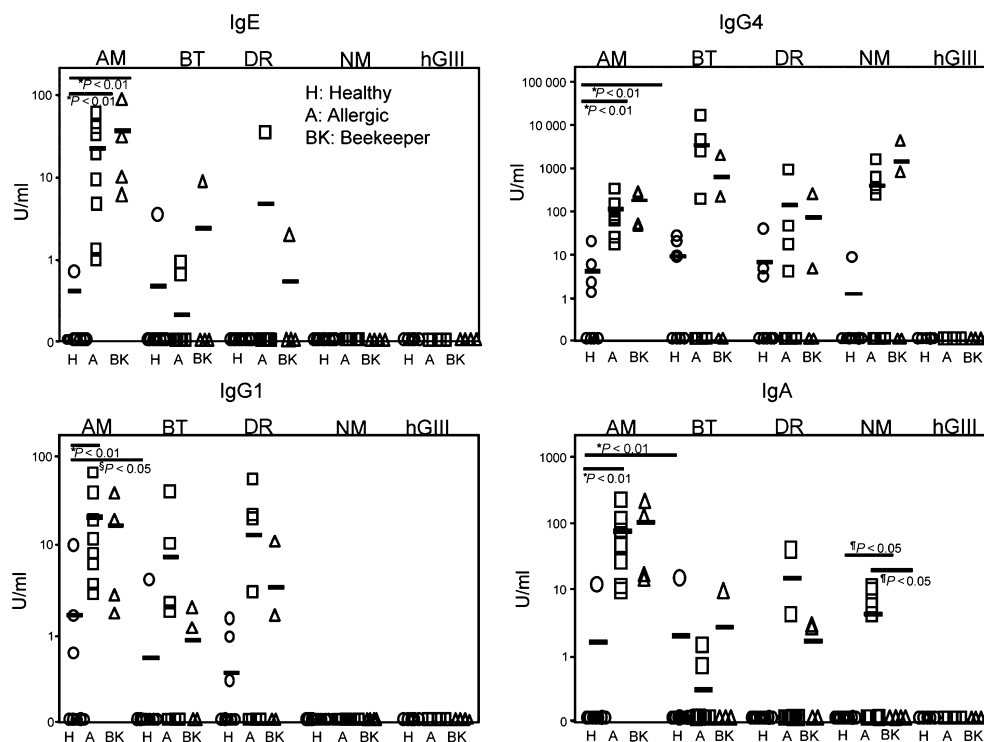


Figure 3 Characterization of the specific antibody response to five different secreted phospholipases A₂ (sPLA₂s). Serum PLA-specific IgE, IgG₄, IgG₁, and IgA Abs were measured against AM, BT, DR, NM, and human group III sPLA₂s. The data shown are the individ-

ual values. Horizontal lines demonstrate the means of each group. [*P < 0.01 (H vs A, H vs BK), §P < 0.05 (H vs BK for IgG₁), ¶P < 0.05 (H vs A, A vs BK for IgA)]. AM, *Apis mellifera*; BT, *Bos taurus*; DR, *Daboia russellii*; NM, *Naja mossambica*.

patients with respect to IL-13 production induced by AM sPLA₂ ($P = 0.020$). IL-13 levels were also elevated in response to NM sPLA₂ in allergic group. In contrast, no significant difference for IL-10 production between groups was observed. Furthermore, IL-4 secretion was undetectable. IFN- γ , IL-13 and IL-10 levels were negative against hGIII sPLA₂ in both controls and allergic patients (Fig. 5).

T-cell proliferation was significantly increased by AM and NM sPLA₂s in allergic patients when compared to controls ($P < 0.031$ and $P < 0.037$, respectively). There was no significant proliferative response to sPLA₂s in controls. No T-cell proliferation was detected against hGIII sPLA₂ in either controls or allergic patients (Fig. 5).

Three TCC against different epitopes of AM sPLA₂ show no cross-reactivity between sPLA₂s. Api m1-induced proliferation of TCC from allergic patients was not observed in response to other sPLA₂s (Fig. 6). Slightly increased T-cell proliferation in response to NM and hGIII sPLA₂s in clones 5.5E7 and 5.6H2 suggests low affinity binding and antigen presentation owing to changes in several amino acids in original T-cell epitope.

Discussion

This study shows that patients, who are allergic to bee venom, possess some cross-reacting antibodies against several

sPLA₂s from diverse sources. However, neither an increase in proliferation nor a specific antibody binding was observed against hGIII sPLA₂ in either allergic or healthy subjects. The data clearly indicate that hGIII sPLA₂ is not recognized as an antigen that stimulates T-cell proliferation and specific antibody binding. This suggests that there is a very strong central tolerance to hGIII sPLA₂ at the level of both T and B cells.

The sPLA₂ found in bee venom represents the most immunogenic component to human beings. The initial response to bee sting results in low-affinity anti-sPLA₂ IgG₁ antibodies, whereas a protective immune response with high-affinity anti-sPLA₂ IgG₄ antibodies is generated after repeated exposures. Thus, IgG₄ has been considered to act as a marker for prolonged antigenic stimuli. In contrast, anti-sPLA₂ IgE antibodies develop in individuals who are allergic to AM (24, 25). Therefore, the primary immune response to sPLA₂ in honeybee venom allergy has a particularly important role, because it may lead to anaphylaxis. Interestingly, we found high levels of specific IgG₄ against sPLA₂s from snake venoms in AM-allergic and hyperimmune subjects. Furthermore, increased levels of IgA to NM venom were also detected in these sera. In light of these observations, it is tempting to speculate that the presence of anti-sPLA₂ antibodies from different origins may account for the cross-tolerance at the regulatory B-cell level in bee venom-allergic and hyperim-

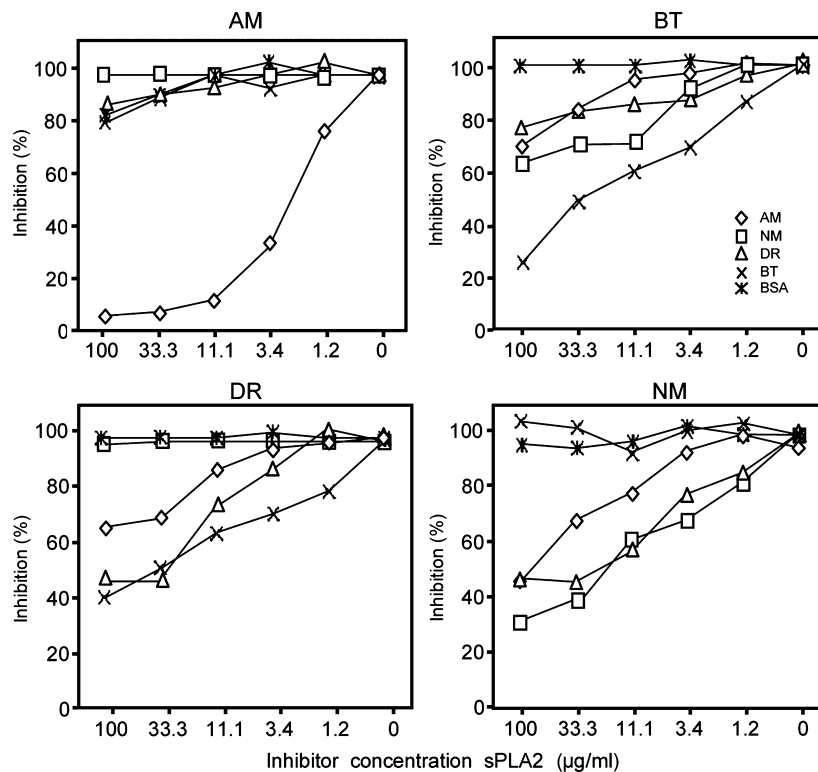


Figure 4 Inhibition ELISA for secreted phospholipases A₂ (sPLA₂s). Plates were coated with sPLA₂s and bovine serum albumin (BSA). Pooled sera from two beekeepers and two allergic individuals were

preincubated for 2 h with the indicated doses of soluble sPLA₂s. The diagrams show the results for IgG₄.

mune subjects. In other words, the presence of anti-sPLA₂ IgG₄ and IgA antibodies from diverse sources may be part of the normal human protective antibody response against cross-sensitization or co-sensitization to different sPLA₂s in allergic subjects and beekeepers. Importantly, cross-reactivity of IgG₄ antibody against other sPLA₂s was determined in AM-allergic and hyperimmune individuals with no apparent exposure to NM and DR snake venoms. We also find that sera from several beekeepers and allergic patients were weakly able to inhibit the enzymatic activity of sPLA₂s.

Shared conformational B-cell epitopes on protein surface may be involved in this process. Thus, cross-reactive epitopes may have only short sequences with high identity in which the adjacent specific pocket is to comprise a immunogenic region responsible for a cross-reacting response. Moreover, it is also indicated that the degree of cross-reactivity between different snake venom sPLA₂s varies (26). This finding demonstrates some similarities of venom antigens among closely related species. However, Reimers et al. reported that IgE-mediated reactions may develop after snake bites. In their study, partial cross-reactivity that may be due to phospholipases was observed in only one subject among four snake-bitten patients who had high specific IgE to both *Vipera* and honey bee venom (27). Lipps et al. (28) also described the antigenic cross-reactivity among the venoms of snakes from major families and venoms from diverse sources, such as bee, scorpion, and toad. In this study, we showed a relatively low

degree of IgE and IgA cross-reactivity against BT, DR, and NM sPLA₂s in allergic and hyperimmune subjects. However, development of IgG₄ Abs reflects a normal immunity to the antigen in exposed individuals, and further studies are needed to confirm cross-reactivity of IgG-binding components and to explain increased IgG₄ Abs to snake venoms in presumably unexposed AM-sensitized subjects.

Another notable finding of our study is that sera of hyperimmune and allergic subjects contain significant amounts of sPLA₂-specific IgG₄ and thereby show cross-reactivity to BT sPLA₂, which is a mammalian but nonhuman sPLA₂. Humans are commonly in contact with BT sPLA₂ via ingestion, but are rarely exposed to snake venoms. Pancreatic sPLA₂ has a well-known role in the intestinal digestion of dietary phospholipids. Although beef is a key source of protein in food consumption, allergy to bovine proteins such as meat is not commonly observed. Meat may contain very low amount of BT sPLA₂, which should be degraded by proteases during digestion. It is shown that bovine IgG was described as a major cross-reacting allergen with other mammalian animal proteins (29). The presence of anti-sPLA₂ IgG₄ antibodies and IgG₄/IgA cross-reactivity against BT sPLA₂ can be explained by the protective role of IgA and IgG₄ that are the part of the mucosal immune response to the antigens consumed via oral route. In support to this view, we have detected low levels of serum-specific IgG₄ and IgA Abs in healthy individuals. However, it should be noted that

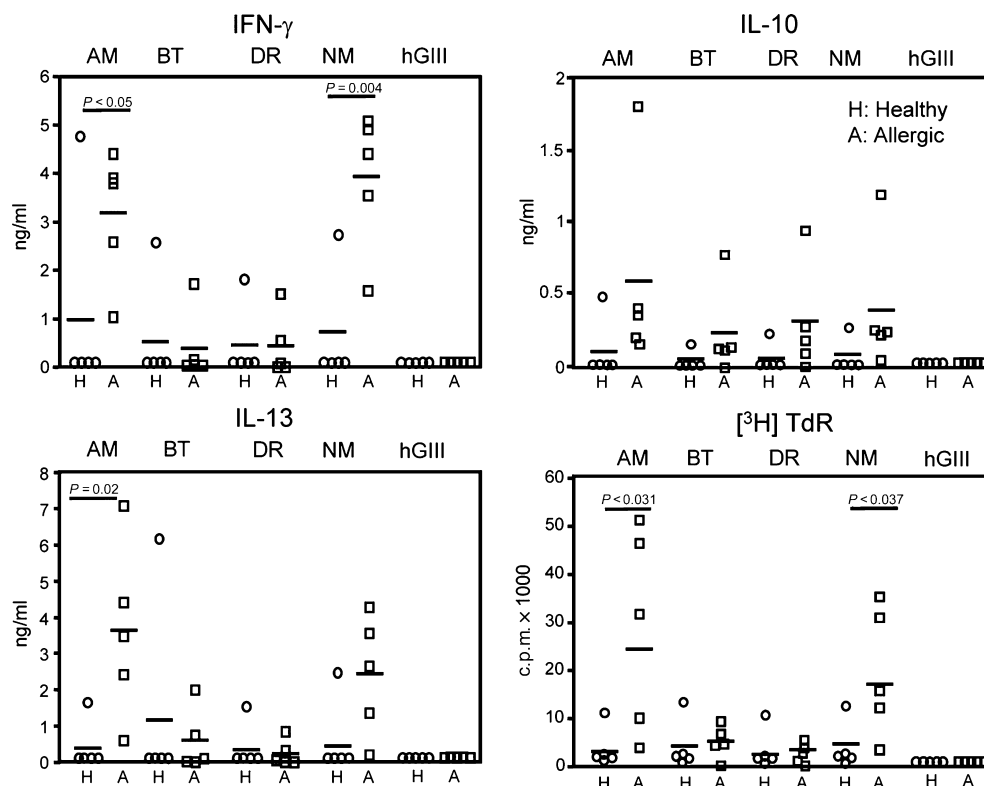


Figure 5 Response of peripheral blood mononuclear cells to different secreted phospholipases A₂ (sPLA₂s). Induction of proliferation and cytokine production in allergic patients and nonallergic controls with 0.3 μ M antigen dose of sPLA₂s. Incorporation of [³H] thymidine (TdR) was measured in cultures at day 5 (cpm, counts per

minute). The levels of IFN- γ , IL-10, and IL-13 in cell supernatants were measured by ELISA after 5 days of culture. Data shown are the individual values. Horizontal lines demonstrate the means for each group. Respective significant values are shown in the figure.

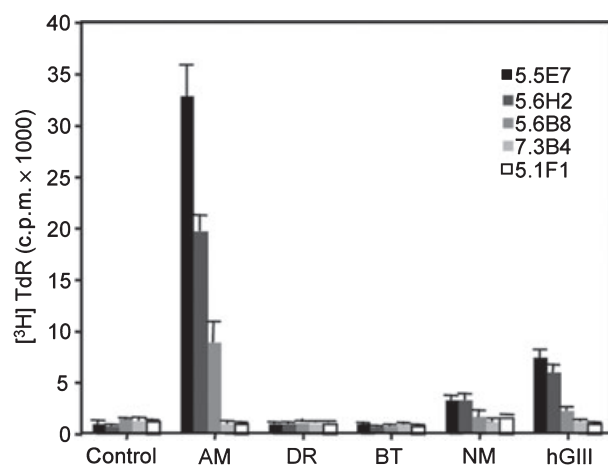


Figure 6 Three T-cell clones (TCC) against different epitopes of AM secreted phospholipases A₂ (sPLA₂) and two control clones 7.3B4 and 5.1F1 were stimulated with different sPLA₂s. [³H] thymidine incorporation was measured on day 3. Irradiated autologous peripheral blood mononuclear cells were used as antigen-presenting cells. There was T-cell cross-reactivity to NM sPLA₂ and human group III sPLA₂ by 5.5E7 and 5.6H2, the AM-sPLA₂ specific TCC. AM, *Apis mellifera*; NM, *Naja mossambica*.

different types of exposure and sensitization or tolerance to environmental antigens may be involved in the development of specific antibody responses.

T-cell help is obviously required for the synthesis of antigen-specific Abs. Here, we showed that NM sPLA₂ induces strong T-cell proliferation. In addition, IFN- γ and less markedly IL-13 production is upregulated by NM sPLA₂ in allergic patients. This finding suggests that this venom sPLA₂ had a more potent stimulating activity than the others at the T-cell level. More pronounced production of IFN- γ was induced by NM sPLA₂, but not bee venom, in allergic patients together with their increased T-cell proliferative response to the same antigen, whereas IL-13 production induced by BV sPLA₂ was higher in allergic patients than in the controls as expected and was associated with T-cell proliferation. In addition, particular cytokine patterns can be modulated by the dose of antigen *in vivo* and do not represent stable phenotypes. Therefore, the modulation of a T-cell cytokine pattern by the dose of antigen may be a driving force for the differential IgE and IgG antibody formation, resulting in either allergy or immunoprotection (24).

Secreted phospholipases A₂ from snake and insect venom and from mammalian pancreas are structurally related enzymes that have been associated with several toxic, patho-

logical, or physiological processes. Marked differences in the toxicity of the sPLA₂s have been determined by Martikainen et al. (30). In their study, sPLA₂ from *Naja cobra* venom displayed more potent cytotoxic effect on MCF-7 cells, yet sPLA₂ from bee venom had no toxic effect. Furthermore, it was shown that *Naja naja* venom sPLA₂ induced apoptotic cell death of human leukemia K562 cells (31). This toxic effect is consistent with the idea that NM sPLA₂ acts more potently than other enzymes to cells. At lower doses, NM may activate T cells better than the others. However, DR sPLA₂ did not significantly stimulate T-cell proliferation and cytokine production despite showing relatively high sequence similarity to NM sPLA₂ and specific antibody responses in the present study. It is known that there is a molecular diversity in venom proteins including sPLA₂s from Russell's viper and Indian cobra venoms. This may explain variations within the antigenicity of snake sPLA₂s on T-cell activation (32).

Evidence from animal models indicated that changes in serum levels of several cytokines were observed with an early increase in IL-6 and IL-10, whereas IFN- γ levels reached higher values in mice injected with some snake venoms (33). Based on these data, it can be postulated that venom components lead to an inflammatory response by activating T cells.

In vitro results that we have observed in regard to T-cell proliferation might be explained by sPLA₂s acting not only as antigens but also as effectors modifying T-cell response. In fact, they may produce lipid mediators or some other cell-sig-

naling cascade that changes the response in terms of cytokine generation (34, 35). The reason for the specific and higher response for T cells isolated from allergic patients may be that they have already been 'primed' by allergy for a more pronounced activation by sPLA₂s. As expected, control subjects did not respond to stimulation with sPLA₂ antigens.

Another important aspect to be considered is the sequence similarities of allergens to human homologs. Although cross-reactivity seems to require more than 70% sequence identity (36), clinically relevant cross-reactivity may occur even at lower sequence identities. Although group III bvPLA₂ has a low sequence identity to other sPLA₂s like BT and NM, its crystal structure has revealed a relatively high level of homology (37), providing a molecular basis for cross-reactivity between the different allergenic sPLA₂s.

We know that a molecular diversity of sPLA₂s exists in both animal venoms and mammalian tissues (1). Our study provides more evidence for distinct cross-reactivity patterns among sPLA₂s from different species at both humoral and T-cell level. However, clinical relevance of these data deserves further investigation. We believe that IgE cross-reactivity may indicate cross-sensitization and allergy, whereas IgG₄ and IgA cross-reactivities may play a role in cross-tolerance or protection.

Conflict of interest

The authors declare no conflict of interest.

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