

Gangliosides inhibit bee venom melittin cytotoxicity but not phospholipase A₂-induced degranulation in mast cells

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ABSTRACT

Sting accident by honeybee causes severe pain, inflammation and allergic reaction through IgE-mediated anaphylaxis. In addition to this hypersensitivity, an anaphylactoid reaction occurs by toxic effects even in a non-allergic person via cytolysis followed by similar clinical manifestations. Auto-injectable epinephrine might be effective for bee stings, but cannot inhibit mast cell lysis and degranulation by venom toxins. We used connective tissue type canine mast cell line (CM-MC) for finding an effective measure that might inhibit bee venom toxicity. We evaluated degranulation and cytotoxicity by measurement of β -hexosaminidase release and MTT assay. Melittin and crude bee venom induced the degranulation and cytotoxicity, which were strongly inhibited by mono-sialoganglioside (G_{M1}), di-sialoganglioside (G_{D1a}) and tri-sialoganglioside (G_{T1b}). In contrast, honeybee venom-derived phospholipase A₂ induced the net degranulation directly without cytotoxicity, which was not inhibited by G_{M1}, G_{D1a} and G_{T1b}. For analysis of distribution of G α_q and G α_i protein by western blotting, lipid rafts were isolated by using discontinuous sucrose gradient centrifuge. Melittin disrupted the localization of G α_q and G α_i at lipid raft, but gangliosides stabilized the rafts. As a result from this cell-based study, bee venom-induced anaphylactoid reaction can be explained with melittin cytotoxicity and phospholipase A₂-induced degranulation. Taken together, gangliosides inhibit the effect of melittin such as degranulation, cytotoxicity and lipid raft disruption but not phospholipase A₂-induced degranulation in mast cells. Our study shows a potential of gangliosides as a therapeutic tool for anaphylactoid reaction by honeybee sting.

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Introduction

Stings by *Hymenopteran* insects are most common as accidents by invertebrates. Two hymenoptera families are particularly dangerous to man: *Apidea* (bees) and *Vespidea* (wasps, hornets, and yellow jackets). *Apis mellifera* has been domesticated for commercial use by bee keepers and now is in a worldwide distribution. *Hymenoptera* venom has potent allergens and various toxins (Antonicelli et al., 2002; Steen et al., 2005). Anaphylaxis is an acute IgE-mediated type I hypersensitivity reaction caused by histamine release from mast cells resulting in acute bronchoconstriction, hypotension, laryngeal constriction, oral edema and rash (Lucke and Thomas, 1983). Bee venom immunotherapy (VIT) is clinically effective and should be recommended for patients with hypersensitivity to prevent future allergic reactions (Ewan, 1998), but the side effects of VIT are more frequent with honeybee than with wasp (Adamic et al., 2009). In addition, an

anaphylactoid reaction occurs by toxic effects, causing death even in a non-allergic person via cytolysis or hemolysis, and cardiac dysfunction (Vetter et al., 1999). Toxic reactions by envenomation from bee stings are symptomatically indistinguishable from allergic ones. This is because mast cell can produce the same mediators and pathological effects. Anaphylaxis to insect stings occurs in 3% of adults and 1% of children (Lieberman et al., 2010). Clinical managements for bee stings include local (ice-packs, topical ointment) and systemic (auto-injectable epinephrine, steroids, anti-histamine, fluid replacement, oxygen therapy) treatment. But these therapeutic modalities cannot prevent pathogenic tissue damage, vasodilatation, smooth muscle constriction and hives caused by histamine leakage and release. Therefore, other toxin-specific treatments for both anti-allergy and anti-cytotoxicity are now required in clinical practices.

Honeybee venom includes phospholipase A₂ (Api m 1), hyaluronidase (Api m 2), acid phosphatase (Api m 3), and melittin (Api m 4) as toxin and allergens (Grunwald et al., 2006). Phospholipase A₂ (PLA₂) represents as super-family of esterase that hydrolyzes sn-2 ester bond of phospholipids releasing lysophospholipid and free fatty acids such as arachidonic acid followed by productions of pro-inflammatory lipid mediators such as prostaglandins and leukotrienes. Hyaluronidase depolymerizes viscous mucopolysaccharide hyaluronic acid to facilitate diffusion of venom in reaction sites. Melittin, cationic and amphiphilic

Abbreviations: CM-MC, canine mastocytoma mast cells; G_{M1}, mono-sialoganglioside; G_{D1a}, di-sialoganglioside; G_{T1b}, tri-sialoganglioside; G α_i , GTP binding regulatory protein α subunit; ROS, reactive oxygen species; PLA₂, phospholipase A₂.

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peptide, has cardio-toxicity by Ca^{2+} overloading (Okamoto et al., 1995) and pore formation in membrane (Raghuraman and Chattopadhyay, 2007). Among other toxins, apamin, basic peptide of 18 amino acid residues cross-linked by two disulfide bridges, is a specific blocker of calcium-dependent potassium channels (Hugues et al., 1982). MCD peptide also triggers histamine release from mast cells through G proteins followed by phospholipase C activation (Mousli et al., 1991). Both melittin and PLA_2 constitute more than half the amount of honeybee venom, therefore, would be effective targets for clinical application to block their toxicity.

We have already found that mastoparan, basic polypeptide from wasp venom, induced the translocation of $\text{G}\alpha_q$ and $\text{G}\alpha_i$ protein from lipid rafts in parallel with the increase of degranulation and ROS generation in mast cells (Nishikawa and Kitani, 2010). Lipid raft is a micro-domain within the plasma membrane enriched with cholesterol, glycolipids, and sphingolipids (Simons and Ikonen, 1997). Honeybee venom melittin might modulate lipid raft which regulates signaling pathways through Fc ϵ RI in mast cells (Dráber and Dráberová, 2002).

In this report, we demonstrated that gangliosides G_{M1} , G_{D1a} and G_{T1b} strongly reduced both melittin- and crude bee venom-caused cytotoxicity by stabilizing G protein α chain and flotillin at lipid raft in mast cells. Honeybee venom-derived PLA_2 directly induced the degranulation from mast cells that was not inhibited by the gangliosides. Gangliosides will be promising for clinical prophylaxis for the cell damage and anaphylactoid reaction by the toxic action of bee venom.

Materials and method

Material. Each chemical was listed with company in parenthesis as follows. IAP (Calbiochem, Darmstadt, Germany); melittin from bee venom, quinacrine, mono-sialoganglioside from bovine brain (G_{M1}), di-sialoganglioside from bovine brain (G_{D1a}), tri-sialoganglioside from bovine brain (G_{T1b}), thiazolyl blue tetrazolium bromide (MTT), N-acetyl- β -D-glucosaminide, PLA_2 purified from porcine pancreases and PLA_2 purified from bee venom (*Apis mellifera*), N-acetyl-neuraminic acid (NANA), lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO); piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and Fura2-AM (Dojindo, Kumamoto, Japan); indomethacin (Wako, Osaka, Japan); anti- $\text{G}\alpha_q$ rabbit polyclonal IgG, (Upstate Lake Placid, NY); anti-flotillin-1 mouse IgG (BD Biosciences, San Jose, CA); anti- $\text{G}\alpha_i$ mouse monoclonal antibody (Chemicon International, Temecula, CA); peroxidase-labeled anti-rabbit IgG (Zymed, South San Francisco, CA); peroxidase-labeled anti-mouse IgG (Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD); synthetic melittin (Alexis Biochemicals, San Diego, CA). YM254890 was kindly gifted from Yamanouchi Co. (Tokyo, Japan). Freeze-dried crude bee venom was kindly gifted from Yamada Bee Farm Co., Ltd (Okayama, Japan).

Cells and buffer. CM-MC were maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) with 10% heat-inactivated FBS at 5% CO_2 37°C as described (Takahashi et al., 2001). PIPES-A buffer consists of 140 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.1% BSA and 10 mM PIPES (pH 7.4). PIPES-ACM was prepared by addition of 0.6 mM MgCl_2 and 1 mM CaCl_2 .

Preparation of crude bee venom. Ten of worker bees collected by a professional bee keeper were anesthetized and kept in a freezer (-20°C). The venom sacs were pulled out by pinching the sting. The venom was eluted in distilled water, lyophilized and stored at -20°C until usage. A bee had 50 μl of venom containing 0.3 mg of solid components. Crude bee venom was also filtered with a 0.22 μm pore size filter for separate experimental use.

Measurements of degranulation. Degranulation was monitored by the release of β -hexosaminidase in supernatants. CM-MC cells were washed twice with PIPES-A by centrifugation at 4°C, 430 $\times$ g for 5 min, and resuspended in PIPES-ACM (2.5×10^6 cells/ml) as described (Sato

et al., 2002). The cells were pre-treated with each reagent at various concentrations for 30 min (G_{M1} , G_{D1a} , G_{T1b} , indomethacin, quinacrine and YM254890) or 2 h (IAP), and were stimulated with crude bee venom or melittin for 10 min, PLA_2 for 30 min at 37°C. The cells were lysed with 0.2% Triton-X-100 to measure total β -hexosaminidase content. After the addition of N-acetyl- β -D-glucosaminide (0.1 M) and glycine-NaOH (0.2 M pH 10.6), supernatants were read at 405 nm with microplate reader Emax (Molecular Device, Sunnyvale, CA). Degranulation (%) was calculated as the percentage of total β -hexosaminidase content in the supernatants using following formula: $100 \times (\text{sample-spontaneous})/(\text{total-spontaneous})$.

Measurements of cytotoxicity. Cytotoxicity was monitored by MTT assay and trypan blue exclusion test. The cell suspensions (2.5×10^6 cells/ml in PIPES-ACM) were stimulated as described above. After the cells were incubated in PBS containing 0.5 mg/ml MTT for 3 h at 37°C, they were sonicated in 40 mM HCl-isopropyl alcohol to dissolve formazan followed by centrifugation at 25000 $\times$ g for 10 min to remove of cell debris. The supernatants were read at 570 nm with spectrophotometer (U-1800, Hitachi, Japan).

Measurements of intracellular free Ca^{2+} concentration. CM-MC cells (5×10^5 cells/ml) were loaded with 2 μM Fura2-AM (Dojindo) for 30 min at 37°C. During measurement of $[\text{Ca}^{2+}]_i$, cells were illuminated alternately with two excitation wavelengths (340 and 380 nm) (Teshima et al., 1997) and fluorescence at 495 nm was measured with spectrophotometer RF-5300 (Shimadzu, Tokyo, Japan).

Isolation of lipid raft from CM-MC. CM-MC cells were pre-treated with ganglioside and stimulated with melittin for 10 min as described. 10^8 cells of CM-MC were washed twice with PIPES-A by centrifugation at 4°C, 430 $\times$ g for 5 min and lysed in 1 ml of lysis buffer containing 50 mM Tris-HCl, 50 mM NaCl, 5 mM EGTA, 1 mM Na_3VO_4 , 5 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 0.1% Triton-X. After sonication for 10 s and shake on ice for 60 min, cell lysates were mixed with 3 ml of 60% sucrose in lysis buffer in a centrifuge tube, and overlaid with 5 ml of 35% sucrose and 4 ml of 5% sucrose. The samples were subjected to ultra-centrifugation at 4°C, 100000 $\times$ g for 24 h by RPS40ST rotor (Hitachi). Each fraction of 1 ml was collected from the top of gradients to the bottom.

Western blotting. Fractions were mixed with equal volume of sample buffer (100 mM Tris, 100 mM DTT, 2% SDS, 10% sucrose and 0.006% bromophenol blue) and subjected to SDS-PAGE using 12.5% acrylamide gels and transferred to PVDF membranes (Hybond P⁺; Amersham Bioscience, Buckinghamshire, UK). The membranes were blocked with 2% skim milk in Tris buffered saline, pH 7.4, containing 0.1% Tween 20 (TBS-T) at 4°C. The membranes were incubated for 2 h at room temperature with primary antibody (1:1000 dilutions for flotillin-1, $\text{G}\alpha_q$ and $\text{G}\alpha_i$) in TBS-T, and then with HRP-conjugated secondary anti-IgG (1:20,000 dilutions) for 1 h. After extensive washing, immunoreactive proteins were detected with ECL plus (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis. Data are expressed as means $\pm$ SD. One-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons in SPSS statistical software version 11. Differences were considered significant when $p < 0.05$.

Results

Direct effect on CM-MC by synthetic melittin and honeybee venom-derived PLA_2

First we investigated the effect of synthetic melittin and phospholipase A_2 on CM-MC. As shown in Fig. 1A, synthetic melittin

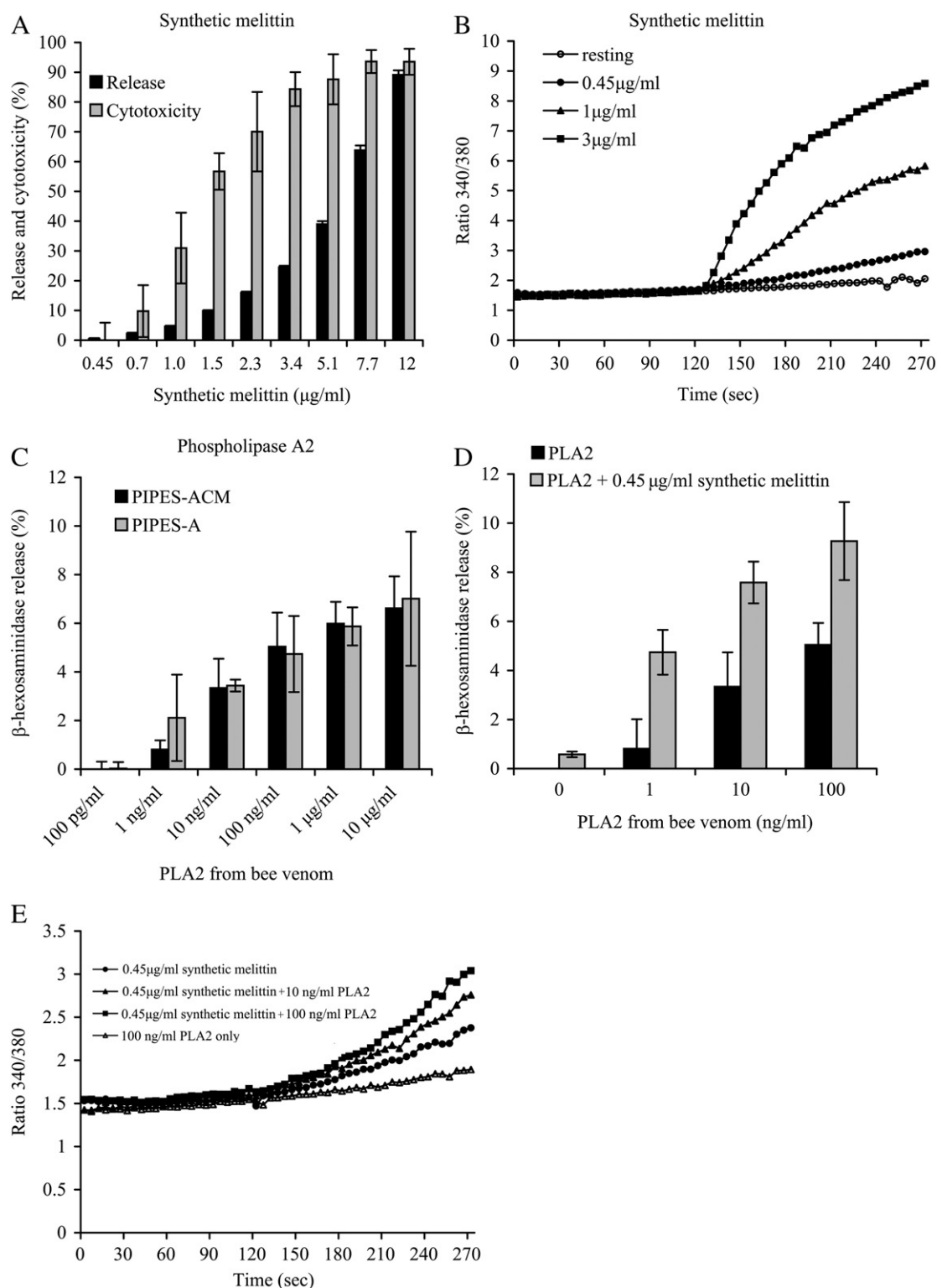


Fig. 1. Direct activation on CM-MC by synthetic melittin and honeybee venom-derived PLA₂. Effect of synthetic melittin on β -hexosaminidase release and MTT cytotoxicity (A), calcium influx (B) in CM-MC. Release by PLA₂ from bee venom in the presence (PIPES-ACM) and absence (PIPES-A) of extracellular calcium. (C). Synergistic activation by co-stimulation with melittin and PLA₂ (D and E). Results are means $\pm$ SD in four independent experiments done in duplicate.

induced 2.2% release at 0.7 μ g/ml and increased the release dose dependently. Cytotoxicity was caused in concentrations above 0.7 μ g/ml as determined by MTT and trypan blue exclusion. Since intracellular calcium ion ($[Ca^{2+}]_i$) plays a critical role for release, we investigated whether melittin increases $[Ca^{2+}]_i$. When cells were stimulated at 1 and 3 μ g/ml, the ratios 340/380 were increased by 6- and 9-fold at 150 s after stimulation, respectively (Fig. 1B). PLA₂

purified from bee venom induced the degranulation dose dependently in the presence and absence of extracellular calcium, and increased by 7% at 10 μ g/ml (Fig. 1C). Since PLA₂ did not show any cytotoxicity up to 100 μ g/ml by MTT and trypan blue exclusion assay, the release was regarded as net degranulation. Porcine pancreas-derived PLA₂ did not induce degranulation (data not shown). With co-stimulation with 0.45 μ g/ml of synthetic melittin, the release by

PLA₂ was synergistically enhanced by 2- or 4-fold as compared with that by PLA₂ alone (Fig. 1D). PLA₂ dose dependently increased calcium signal as monitored by ratio 340/380 (Fig. 1E).

Effect of gangliosides on cytotoxicity by melittin and degranulation by venom PLA₂ in CM-MC

The release from CM-MC by 3 µg/ml of synthetic melittin (cytotoxic concentration) was reduced by 10 µM of mono-sialoganglioside (G_{M1}) and di-sialoganglioside (G_{D1a}) from 22.5 ± 3.71% to 2.23 ± 1.39 and 3.22 ± 2.31%, respectively (Fig. 2A). Tri-sialoganglioside (G_{T1b}) also reduced the release from 27.1 ± 2.17% to 2.17 ± 1.38% (Fig. 2A). Quinacrine, known as PLA₂ inhibitor, also reduced the release from 23.7 ± 4.07% to 11.2 ± 3.68% at 30 µM (Fig. 2A). On the other hand, indomethacin, inhibitor of cyclooxygenase, did not decrease the release. YM254890, selective Gα_q inhibitor that blocks the exchange step of GDP for GTP (Takasaki et al., 2004), showed no effects. IAP (pertussis toxin), which uncouples G proteins from receptors by

catalyzing ADP-ribosylation of α subunits of the heterotrimeric G proteins, did not reduce the release. Cytotoxicities were decreased by 10 µM of G_{M1} and G_{D1a} from 57.9 ± 9.69% to 25.1 ± 7.70% and 18.2 ± 8.60%, respectively (Fig. 2B). Similarly G_{T1b} decreased the cytotoxicity from 61.6 ± 5.96% to 22.9 ± 10.7% at 10 µM (Fig. 2B).

These gangliosides (30 µM) slightly enhanced the release by PLA₂ but not significantly (Fig. 2C). *N*-acetylneuraminic acid (NANA), major sialic acid of ganglioside, did not reduce the release induced by PLA₂. Quinacrine decreased the release slightly but did not significantly (*P* = 0.10).

Gangliosides reduced the cytotoxicity and degranulation by purified melittin from bee venom

As shown in Fig. 3A, melittin increased the release dose dependently and simultaneously caused cytotoxicity in concentrations above 0.7 µg/ml. When cells were stimulated with 0.45 µg/ml of melittin (non-cytotoxic concentration), gangliosides G_{M1}, G_{D1a}, G_{T1b},

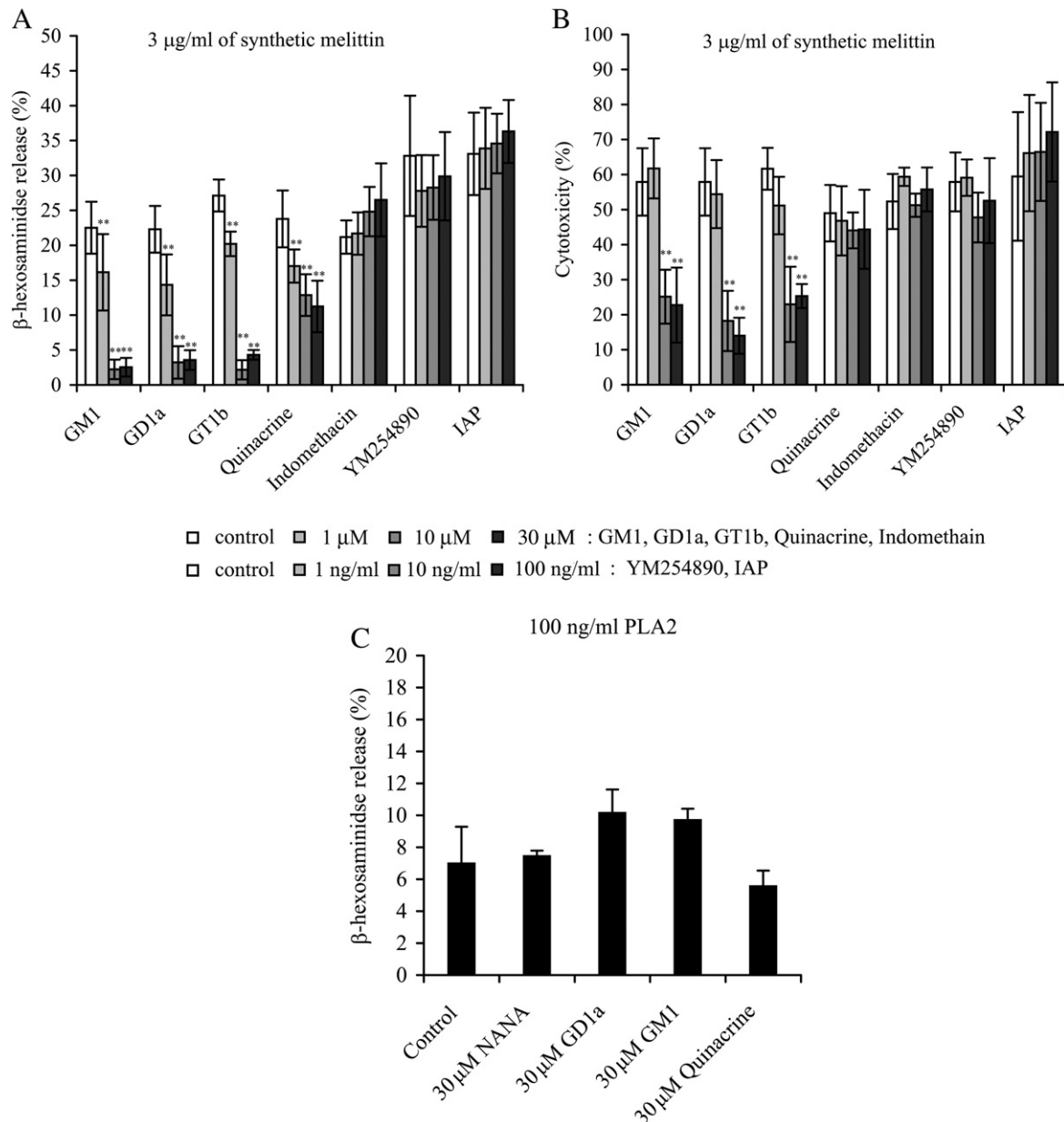


Fig. 2. Effect of gangliosides on CM-MC by synthetic melittin and honeybee venom-derived PLA₂. Effect of gangliosides and other compounds on melittin-induced release (A) and cytotoxicity (B). Effect of gangliosides and quinacrine on PLA₂-induced release (C). Results are means ± SD in four independent experiments done in duplicate. **P* < 0.05; ***P* < 0.01.

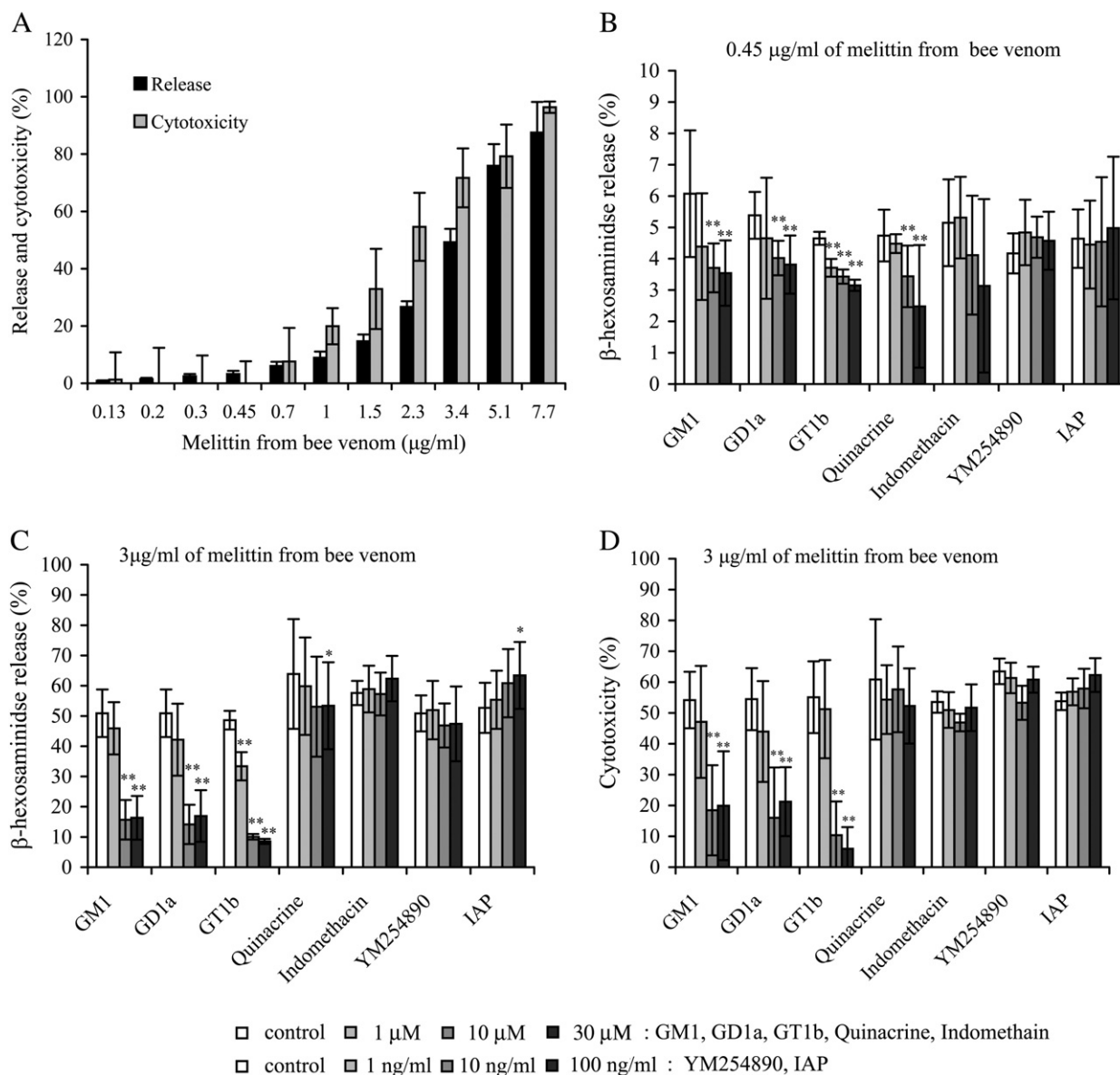


Fig. 3. Effect of ganglioside on cytotoxicity and release caused by purified melittin from honeybee venom. Dose–response of cytotoxicity and release by purified melittin from honeybee venom (A). Effect of gangliosides on the release by 0.45 µg/ml melittin (B), or on the release (C) and cytotoxicity (D) by 3 µg/ml melittin. Results are means $\pm$ SD in four independent experiments done in duplicate. * P <0.05; ** P <0.01.

quinacrine and indomethacin attenuated the release (Fig. 3B). Even when cells were stimulated with 3 µg/ml of melittin (cytotoxic concentration), both G_{M1} and G_{D1a} obviously reduced the release from $50.9 \pm 7.85\%$ to $5.7 \pm 6.54\%$ and $14.1 \pm 6.47\%$ (Fig. 3C). G_{T1b} similarly attenuated the release from $48.6 \pm 3.08\%$ to $10.0 \pm 0.91\%$ (Fig. 3C). Cytotoxicities were obviously reduced by 10 µM of G_{M1} , G_{D1a} and G_{T1b} from $54.5 \pm 9.15\%$ to $18.4 \pm 14.6\%$, $15.9 \pm 16.3\%$ and $10.3 \pm 10.9\%$, respectively (Fig. 3D). Quinacrine slightly attenuated the release from $63.8 \pm 18.1\%$ to $53.0 \pm 16.5\%$ (Fig. 3C), but did not affect the cytotoxicity (Fig. 3D). Indomethacin, YM254890 and IAP showed no effects on both the release and cytotoxicity (Fig. 3C and D). Gangliosides G_{M1} , G_{D1a} and G_{T1b} also reduced the effect of melittin filtered with 0.22 µm pore size (data not shown).

Gangliosides stabilized G protein α and flotillin-1 at lipid raft

We investigated the modification of melittin on the localization of G protein α -chain at lipid raft. Cell lysates were subjected to discontinuous sucrose density gradients followed by detections in

each fraction by western blotting. $G\alpha_q$ specific bands were detected as 42 kDa protein in 6th fraction in resting condition (Fig. 4A, upper), whereas melittin potently reduced the density of $G\alpha_q$ in the same fraction, but G_{D1a} and G_{M1} recovered its level into that of resting condition (Fig. 4A, lower). $G\alpha_i$ specific bands were also detected as 42 kDa protein in 6th fraction (Fig. 4B, upper). Melittin also decreased the $G\alpha_i$ specific band in cytosolic 6th fraction where gangliosides recovered the level of $G\alpha_i$ protein (Fig. 4B, lower). As shown in Fig. 4C, flotillin-1, lipid raft marker, was identified in 6th fraction (48 kDa). Gangliosides G_{D1a} and G_{M1} recovered density of flotillin-1 band at lipid raft which had become thinner by 3 µg/ml melittin. Thus, gangliosides stabilized signaling molecules G protein α chain and flotillin-1 in their distribution at lipid raft.

Gangliosides inhibited the toxic and releasing effects of crude honeybee venom in CM-MC

Crude bee venom induced the release by 5.27% at 0.13 µg/ml and increased its degree dose dependently. Crude venom was also

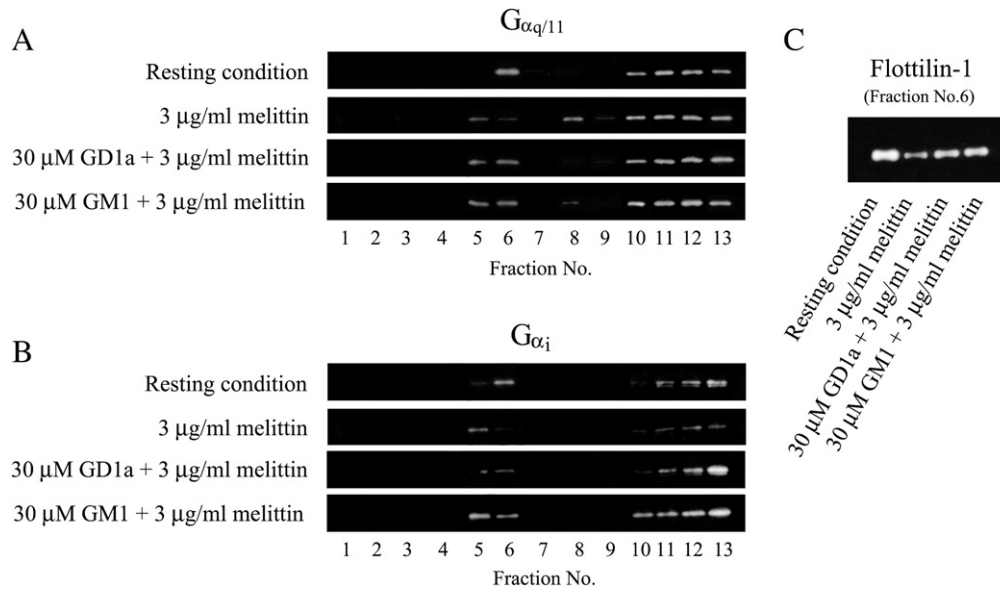


Fig. 4. Modification of gangliosides on the localization of G proteins at lipid raft in CM-MC. Cells were pre-treated with 30 μ M of G_{D1a} and G_{M1} , stimulated with 3 μ g/ml of melittin from bee venom, fractionated and separated into 13 fractions. Signaling molecules $G_{\alpha q/11}$ (A) and $G_{\alpha i}$ (B) in all fractions and lipid raft marker flotillin-1 in the 6th fraction (C) were analyzed by western blotting. The data are representative of two independent experiments with similar results.

cytotoxic in concentrations above 1.5 μ g/ml (Fig. 5A). Filtered endotoxin-free bee venom was evaluated for potency of degranulation and was found to show the same results as non-filtered one (Fig. 5A, white column shown below a horizontal line). We also confirmed that 5 μ g/ml of lipopolysaccharide (LPS), molecular entity of endotoxin effect, did not induce nor enhance any degranulation by filtered crude bee venom (data not shown).

As shown in Fig. 5B, gangliosides G_{M1} , G_{D1a} and G_{T1b} reduced the release from $58.7 \pm 13.0\%$ to $18.3 \pm 4.00\%$, from $53.4 \pm 11.0\%$ to $15.9 \pm 6.82\%$ and from $54.9 \pm 13.1\%$ to $22.7 \pm 2.16\%$ at concentration of 30 μ M, respectively. Cytotoxicity was also attenuated by these gangliosides dose dependently. Notably, G_{T1b} completely inhibited cytotoxicity in concentrations above 3 μ M and showed the most potent inhibitory action among three gangliosides. The number of sialic acid affected the efficacy; the potency was $G_{T1b} > G_{D1a} > G_{M1}$ (Fig. 3C, D and Fig. 5C). Gangliosides G_{M1} , G_{D1a} and G_{T1b} showed similar inhibitory effects on the degranulation by filtered bee venom (80% inhibition) (data not shown).

Discussion

Bee venom causes the sign and symptoms such as severe pain, local inflammation and systemic allergic reaction through IgE-mediated type I anaphylaxis by even a single sting. In addition, anaphylactoid reaction by envenomation shows similar manifestations in population with no previous sting history. In both IgE and non-IgE reaction, mast cells rapidly release histamine, proteases and cytokines via exocytosis or leakage.

Canine cutaneous mastocytoma cell (CM-MC), obtained from canine mastocytoma, is both tryptase- and chymase-positive (Takahashi et al., 2001). CM-MC is also rat peritoneal connective tissues type which is activated with compound 48/80 (Nishikawa et al., 2007) and has been studied for melittin effect (Ishimoto et al., 1996). The domesticated dog lives in similar environment, physical activity, housing and food to humans, and shows anaphylactic shock by bee sting (Walker et al., 2005). It is expected that the dog would be a help for understanding pathogenesis of human disease by new information of a canine genome sequence (Parker et al., 2010). Thus, CM-MC is a good in vitro cell-based model for study of bee sting.

Melittin, the most abundant constituent of honeybee venom (Habermann, 1972), is an amphiphilic peptide of 26 amino acids (Gly-

Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln) with a hydrophobic N-terminal region (1–20 amino acids) and C-terminal region (21–26) with positively charged amino acids (Terra et al., 2007). Its basic C-terminal hexapeptide is associated with negatively charged membrane phospholipids, and the insertion of hydrophobic N-terminal region into bilayer structure causes disruption of membrane lipids (Dawson et al., 1978).

LC_{50} s of melittin from bee venom and crude bee venom were 2.15 and 4.54 μ g/ml, respectively (Figs. 3A and 5A). This is in agreement with the report that half of bee venom consists of melittin in terms of dry weight (Pacáková et al., 1995). LC_{50} of synthetic melittin was 1.22 μ g/ml, showing stronger toxicity than that of melittin from bee venom. It is thought that melittin from bee venom contains some precursors or degradation products without functions (Matysiak et al., 2011).

Furthermore, melittin is known to activate $G_{\alpha i}$ and $G_{\alpha o}$ proteins directly in vitro cell-free system (Higashijima et al., 1990) or small G rho protein (Koch et al., 1992). In our in vivo cell-based study, both YM254890 and IAP did not inhibit the release and cytotoxicity (Figs. 2A, B and 3A, B). Quinacrine minimally attenuated β -hexosaminidase release but did not inhibit cytotoxicity by melittin, (Figs. 2A, B and 3), suggesting the partial involvement of PLA_2 activation, i.e., lysophospholipid or arachidonic cascade-derived metabolites. Inhibition of melittin-caused intracellular cytosolic PLA_2 activation may not be sufficient to reduce allergic reaction in vivo (Hartman et al., 1991).

Secretory PLA_2 s (s PLA_2 s) are classified into group IB (GIB), GII (subgroups A–F), GIII, GV, GX and GXII (subgroups A–B) (Lambeau and Gelb, 2008). Bee venom-derived group III s PLA_2 consists of 135 amino acids and has five disulfide bridges, including a well-defined Ca^{2+} loop, three large α -helices, and a β -wing-like structure (Valentin and Lambeau, 2000). Bee venom phospholipase A2, one of the major allergen (Api m 1) in IgE system (Blaser et al., 1998), has been reported to produce leukotrienes but not to release histamine from human basophils (Mustafa et al., 2008). We used the same source of venom secretory PLA_2 as this, implicating no contaminations or endotoxin which could cause false positivity. Another laboratory reported that allergen PLA_2 (10–100 μ g/ml) induced IgE-independent IL4 production (Dudler et al., 1995) but also leaked false positive histamine concomitant with LDH release (cytolytic marker) in

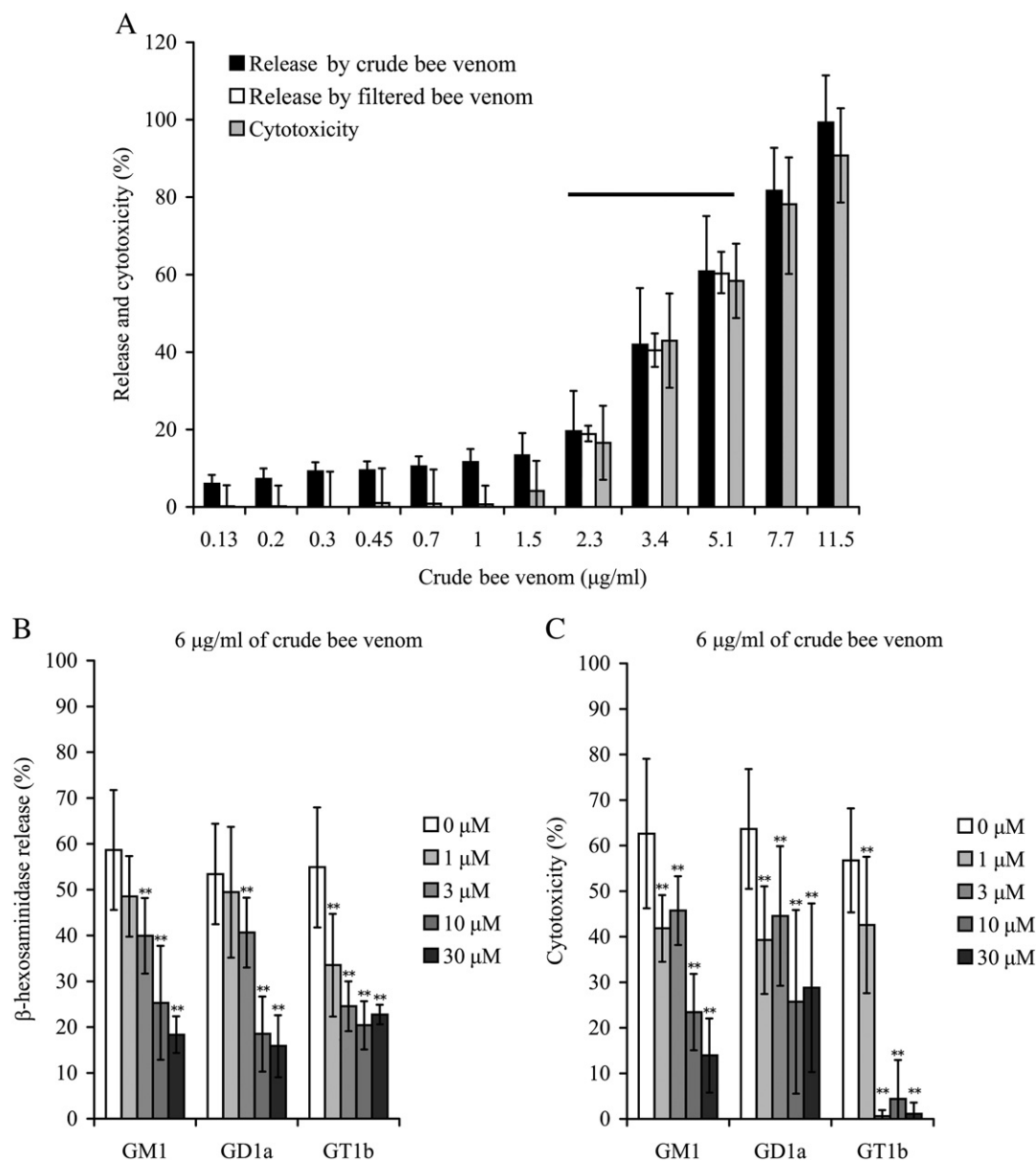


Fig. 5. Crude honeybee venom-induced cytotoxicity and release in CM-MC. Dose-response of cytotoxicity and release by crude honeybee venom (A). Effects of gangliosides on release (B) and cytotoxicity (C). The release by filtered bee venom is shown with white column below a horizontal line. Results are means $\pm$ SD in four independent experiments done in duplicate. * $P < 0.05$; ** $P < 0.01$.

transfected RBL cell (Machado et al., 1996). In our experiments, bee venom sPLA₂ directly induced the net degranulation at relatively low concentration (1 ng/ml) in mast cells without any cytotoxicity (Fig. 1C). And it is thought that the release by crude bee venom in concentrations above 1.5 $\mu\text{g/ml}$ was reflecting both the net degranulation and leakage by cytotoxicity. As for crude bee venom, the net amount of PLA₂ at 0.13 $\mu\text{g/ml}$ of crude venom is equal to 20 ng/ml since PLA₂ accounts for 15% of bee venom in dry weight (Pacáková et al., 1995). Therefore, the release by 0.13–1 $\mu\text{g/ml}$ of crude bee venom is comparable with venom sPLA₂ (Fig. 1D vs. Fig. 5A). Thus, this is the most definitive report that bee venom-derived PLA₂ directly causes the release from mast cells but not through IgE antibody to PLA₂ (Api m 1).

The mechanism still remains undetermined. But PLA₂ from bee venom is thought to bind to N-type sPLA₂ receptor (Nicolas et al., 1997) and can induce activation in dendritic cell (Ramoner et al., 2005). And snake venom PLA₂ induces exocytosis through vesicle-membrane fusion by two hydrolyzed products (lysophospholipids

and fatty acid) at synapse (Rigoni et al., 2005). Arachidonic acid penetrates into hydrophobic grooves between the amphipathic helices of syntaxin, changing the conformation and promoting SNARE complex formation (Darios et al., 2007).

We herein showed that the combination of melittin and PLA₂ augmented the release and $[\text{Ca}^{2+}]_i$ (Fig. 1D and E). This suggests a synergistic toxicity by two complementary mechanisms: cytolytic melittin and fusogenic phospholipase A₂. Hemolytic synergy is well documented among numerous pore-forming toxins; lethal action of yellow jackets' venom requires a synergistic action of mastoparan and phospholipase A₁ (King et al., 2003).

Interestingly enough, the signaling molecules G α_i and G α_q specific bands detected at lipid raft were disrupted by melittin but were stabilized by gangliosides (Fig. 4A and B). From toxicological point of view, cell surface gangliosides play an important role in toxin entry into cell. GM1 and another glycolipid globotriasyl-ceramide serve also as membrane receptors for cholera toxin (Heyning, 1974) and shiga toxin (Jacewicz et al., 1986), respectively. Cholera toxin CTB binds to

cell surface gangliosides G_{M1} in lipid rafts and accelerates disease progression in lupus-prone mice by promoting their aggregation (Deng and Tsokos, 2008).

In IgE system using RBL-2H3, exogenous gangliosides (Dráberová et al., 2003), or ganglioside G_{D1a} specific monoclonal antibody AA4 (Oliver et al., 1992) inhibited FcεRI mediated degranulation. In our non-IgE system, gangliosides significantly reduced the cytotoxicity by melittin. As far as we know, this is the first report showing that G_{α_i} and G_{α_q} protein are translocated from lipid rafts by melittin and that gangliosides inhibit these changes in connective tissue type mast cells. Ganglioside is bio-synthesized by sialyl-transferases which convert lactosylceramide into ganglioside through stepwise addition of sialic acid residues to the galactose unit, and a-, b-, and c-series of ganglioside through sequential addition of N-acetylgalactosamine, galactose, and sialic acid residues. Ganglioside has an amphiphilicity of hydrophobic ceramide and hydrophilic oligosaccharide chain and might decrease membrane fluidity by liquid-ordered and liquid-disordered phase change. Further bio-physical study on interaction among bee venom, membrane lipid composition and signaling apparatus will be required.

In conclusion, we show here that crude bee venom induced both degranulation and cytotoxicity; the former by PLA₂ and the latter by melittin. In addition, exogenously added gangliosides G_{M1} , G_{D1a} and G_{T1b} decreased them by stabilizing G protein at lipid raft where they might modulate the membrane topology and signaling network. Industrial extractions from buffalo milk or productions such as synthesis of novel G_{M2} analogs are recently developed (Fuse et al., 2006). Melittin-induced paw edema in mice model was not inhibited by any known anti-allergic drugs to date (Hartman et al., 1991). Auto-injectable epinephrine is an effective therapeutic management for bee stings, but cannot inhibit mast cell lysis and degranulation induced by venom toxins. Although the efficacy has to be addressed in animal or human study, we propose gangliosides as therapeutic tool or clinical prophylaxis for not only hypersensitivity but also anaphylactoid reaction by bee sting.

Conflict of interest statement

There are no conflicts of interest.

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References

- Adamic, K., Zidarn, M., Bajrovic, N., Erzen, R., Kopac, P., Music, E., 2009. The local and systemic side-effects of venom and inhaled-allergen subcutaneous immunotherapy. *Wien. Klin. Wochenschr.* 121, 357–360.
- Antonicelli, L., Bilò, M., Bonifazi, F., 2002. Epidemiology of Hymenoptera allergy. *Curr. Opin. Allergy Clin. Immunol.* 2, 341–346.
- Blaser, K., Carballido, J., Faith, A., Cramer, R., Akdis, C., 1998. Determinants and mechanisms of human immune responses to bee venom phospholipase A2. *Int. Arch. Allergy Immunol.* 117, 1–10.
- Darios, F., Connell, E., Davletov, B., 2007. Phospholipases and fatty acid signalling in exocytosis. *J. Physiol.* 585, 699–704.
- Dawson, C., Drake, A., Helliwell, J., Hider, R., 1978. The interaction of bee melittin with lipid bilayer membranes. *Biochim. Biophys. Acta* 510, 75–86.
- Deng, G., Tsokos, G., 2008. Cholera toxin B accelerates disease progression in lupus-prone mice by promoting lipid raft aggregation. *J. Immunol.* 181, 4019–4026.
- Dráber, P., Dráberová, L., 2002. Lipid rafts in mast cell signaling. *Mol. Immunol.* 38, 1247–1252.
- Dráberová, L., Dudková, L., Boubelík, M., Tolarová, H., Smíd, F., Dráber, P., 2003. Exogenous administration of gangliosides inhibits FcεRI-mediated mast cell degranulation by decreasing the activity of phospholipase C gamma. *J. Immunol.* 171, 3585–3593.
- Dudler, T., Machado, D., Kolbe, L., Annand, R., Rhodes, N., Gelb, M., et al., 1995. A link between catalytic activity, IgE-independent mast cell activation, and allergenicity of bee venom phospholipase A2. *J. Immunol.* 155, 2605–2613.

- Ewan, P., 1998. Venom allergy. *BMJ* 316, 1365–1368.
- Fuse, T., Ando, H., Imamura, A., Sawada, N., Ishida, H., Kiso, M., et al., 2006. Synthesis and enzymatic susceptibility of a series of novel GM2 analogs. *Glycoconj. J.* 23, 329–343.
- Grunwald, T., Bockisch, B., Spillner, E., Ring, J., Bredehorst, R., Ollert, M., 2006. Molecular cloning and expression in insect cells of honeybee venom allergen acid phosphatase (Api m 3). *J. Allergy Clin. Immunol.* 117, 848–854.
- Hartman, D.A., Tomchek, L.A., Lugay, J.R., Lewin, A.C., Chau, T.T., Carlson, R.P., 1991. Comparison of antiinflammatory and anti-allergic drugs in the melittin- and D49 PLA2-induced mouse paw edema models. *Agents Actions* 34, 84–88.
- Habermann, E., 1972. Bee and wasp venoms. *Science* 177, 314–322.
- Heyningen, S.V., 1974. Cholera toxin: interaction of subunits with ganglioside GM1. *Science* 183, 656–657.
- Higashijima, T., Burnier, J., Ross, E., 1990. Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. *J. Biol. Chem.* 265, 14176–14186.
- Hugues, M., Romey, G., Duval, D., Vincent, J., Lazdunski, M., 1982. Apamin as a selective blocker of the calcium-dependent potassium channel in neuroblastoma cells: voltage-clamp and biochemical characterization of the toxin receptor. *Proc. Natl. Acad. Sci. USA* 79, 1308–1312.
- Ishimoto, T., Akiba, S., Sato, T., 1996. Importance of the phospholipase D-initiated sequential pathway for arachidonic acid release and prostaglandin D2 generation by rat peritoneal mast cells. *J. Biochem.* 120, 616–623.
- Jacewicz, M., Clausen, H., Nudelman, E., Donohue-Rolfé, A., Keusch, G., 1986. Pathogenesis of shigella diarrhea: XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J. Exp. Med.* 163, 1391–1404.
- King, T., Jim, S., Wittkowski, K., 2003. Inflammatory role of two venom components of yellow jackets (*Vespula vulgaris*): a mast cell degranulating peptide mastoparan and phospholipase A1. *Int. Arch. Allergy Immunol.* 131, 25–32.
- Koch, G., Habermann, B., Mohr, C., Just, I., Aktories, K., 1992. ADP-ribosylation of rho proteins is inhibited by melittin, mast cell degranulating peptide and compound 48/80. *Eur. J. Pharmacol.* 226, 87–91.
- Lambeau, G., Gelb, M., 2008. Biochemistry and physiology of mammalian secreted phospholipases A2. *Annu. Rev. Biochem.* 77, 495–520.
- Lieberman, P., Nicklas, R., Oppenheimer, J., Kemp, S., Lang, D., Bernstein, D., et al., 2010. The diagnosis and management of anaphylaxis practice parameter: 2010 update. *J. Allergy Clin. Immunol.* 126, 477–480.e471–442.
- Lucke, W., Thomas, H.J., 1983. Anaphylaxis: pathophysiology, clinical presentations and treatment. *J. Emerg. Med.* 1, 83–95.
- Machado, D., Horton, D., Harrop, R., Peachell, P., Helm, B., 1996. Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *Eur. J. Immunol.* 26, 2972–2980.
- Matysiak, J., Schmelzer, C.E., Neubert, R.H., Kokot, Z.J., 2011. Characterization of honeybee venom by MALDI-TOF and nanoESI-QqTOF mass spectrometry. *J. Pharm. Biomed. Anal.* 54, 273–278.
- Mousli, M., Bronner, C., Bueb, J., Landry, Y., 1991. Evidence for the interaction of mast cell-degranulating peptide with pertussis toxin-sensitive G proteins in mast cells. *Eur. J. Pharmacol.* 207, 249–255.
- Mustafa, F., Ng, F., Nguyen, T., Lim, L., 2008. Honeybee venom secretory phospholipase A2 induces leukotriene production but not histamine release from human basophils. *Clin. Exp. Immunol.* 151, 94–100.
- Nicolas, J., Lin, Y., Lambeau, G., Ghomashchi, F., Lazdunski, M., Gelb, M., 1997. Localization of structural elements of bee venom phospholipase A2 involved in N-type receptor binding and neurotoxicity. *J. Biol. Chem.* 272, 7173–7181.
- Nishikawa, H., Wakano, K., Kitani, S., 2007. Inhibition of NADPH oxidase subunits translocation by tea catechin EGCG in mast cell. *Biochem. Biophys. Res. Commun.* 362, 504–509.
- Nishikawa, H., Kitani, S., 2010. Inhibitory effect of ganglioside on mastoparan-induced cytotoxicity and degranulation in lipid raft of connective tissue type mast cell. *J. Biochem. Mol. Toxicol.* [Epub ahead of print] PubMed PMID: 21077213.
- Okamoto, T., Isoda, H., Kubota, N., Takahata, K., Takahashi, T., Kishi, T., et al., 1995. Melittin cardiotoxicity in cultured mouse cardiac myocytes and its correlation with calcium overload. *Toxicol. Appl. Pharmacol.* 133, 150–163.
- Oliver, C., Sahara, N., Kitani, S., Robbins, A., Mertz, L., Siraganian, R., 1992. Binding of monoclonal antibody AA4 to gangliosides on rat basophilic leukemia cells produces changes similar to those seen with Fc epsilon receptor activation. *J. Cell Biol.* 116, 635–646.
- Pacáková, V., Stulik, K., Hau, P.T., Jelinek, I., Vins, I., Sykora, D., 1995. Comparison of high-performance liquid chromatography and capillary electrophoresis for the determination of some bee venom components. *J. Chromatogr. A* 700, 187–193.
- Parker, H.G., Shearin, A.L., Ostrander, E.A., 2010. Man's best friend becomes biology's best in show: genome analyses in the domestic dog. *Annu. Rev. Genet.* 44, 309–336.
- Raghuraman, H., Chattopadhyay, A., 2007. Melittin: a membrane-active peptide with diverse functions. *Biosci. Rep.* 27, 189–223.
- Ramoner, R., Putz, T., Gander, H., Rahm, A., Bartsch, G., Schaber, C., Thurnher, M., 2005. Dendritic-cell activation by secretory phospholipase A2. *Blood* 105, 3583–3587.
- Rigoni, M., Caccin, P., Gschmeissner, S., Koster, G., Postle, A., Rossetto, O., et al., 2005. Equivalent effects of snake PLA2 neurotoxins and lysophospholipid-fatty acid mixtures. *Science* 310, 1678–1680.
- Sato, Y., Teshima, R., Nakamura, R., Sasaki, N., Morita, Y., Sawada, J., Kitani, S., 2002. IgG-mediated signal transduction in canine mastocytoma-derived cells. *Int. Arch. Allergy Immunol.* 129, 305–313.

- Simons, K., Ikonen, E., 1997. Functional rafts in cell membranes. *Nature* 387, 569–572.
- Steen, C., Janniger, C., Schutzer, S., Schwartz, R., 2005. Insect sting reactions to bees, wasps, and ants. *Int. J. Dermatol.* 44, 91–94.
- Takahashi, T., Kitani, S., Nagase, M., Mochizuki, M., Nishimura, R., Morita, Y., Sasaki, N., 2001. IgG-mediated histamine release from canine mastocytoma-derived cells. *Int. Arch. Allergy Immunol.* 125, 228–235.
- Takasaki, J., Saito, T., Taniguchi, M., Kawasaki, T., Moritani, Y., Hayashi, K., Kobori, M., 2004. A novel Galphaq/11-selective inhibitor. *J. Biol. Chem.* 279, 47438–47445.
- Terra, R., Guimarães, J., Verli, H., 2007. Structural and functional behavior of biologically active monomeric melittin. *J. Mol. Graph. Model* 25, 767–772.
- Teshima, R., Saito, Y., Ikebuchi, H., Rajiva De Silva, N., Morita, Y., Nakanishi, M., et al., 1997. Effect of an ectokinase inhibitor, K252b, on degranulation and Ca^{2+} signals of RBL-2H3 cells and human basophils. *J. Immunol.* 159, 964–969.
- Valentin, E., Lambeau, G., 2000. What can venom phospholipases A(2) tell us about the functional diversity of mammalian secreted phospholipases A(2)? *Biochimie* 82, 815–831.
- Vetter, R., Visscher, P., Camazine, S., 1999. Mass envenomations by honey bees and wasps. *West. J. Med.* 170, 223–227.
- Walker, T., Tidwell, A., Rozanski, E., DeLaforcade, A., Hoffman, A., 2005. Imaging diagnosis: acute lung injury following massive bee envenomation in a dog. *Vet. Radiol. Ultrasound* 46, 300–303.