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The Inhibitory Mechanism of Aloe Glycoprotein (NY945) on the Mediator Release in the Guinea Pig Lung Mast Cell Activated with Antigen-Antibody Complexes

Jai Youl Ro[1], Byung Chul Lee[2], Myung Hee Chung[3], Seung Ki Lee[4], Chung Ki Sung[5], Kyung Hwan Kim[1], and Young In Park[2]

[1]Departments of Pharmacology, Yonsei University College of Medicine, Seoul 120−752, Korea; [2]Graduate School of Biotechnology Korea university, Seoul 136−701, Korea; [3]Department of Pharmacology, Seoul National University College of Medicine, Seoul 110−799, Korea; [4]Department of Biochemistry, Seoul National University College of Pharmacy, Seoul 151−742, Korea; [5]Department of Pharmaceutics, Chunnam University College of Pharmacy, Chunnam 500−757, Korea

It has been reported that the glycoprotein extracted from Aloe has strong anti-inflammatory response. However, there has been no research report yet about the effect of Aloe on allergic hypersensitivity reactivity. By using guinea pig lung mast cells, this study aimed to examine the effects of Aloe glycoprotein (NY945) on the mediator releases caused by mast cell activation, and also aimed to assess the effects of NY945 on the mechanism of mediator releases in the mast cell activation. We partially purified mast cell from guinea pig lung tissues by using the enzyme digestion, the rough and the discontinuous density percoll gradient method. Mast cells were sensitized with IgG_1 (anti-OA) and challenged with ovalbumin. Histamine was assayed by fluorometric analyzer, leukotrienes by radioimmunoassay. The phospholipase D activity was assessed by the production of labeled phosphatidylalcohol. The amount of mass 1, 2-diacylglycerol (DAG) was measured by the [^{3}H]DAG produced when prelabeled with [^{3}H]myristic acid. The phospholipid methylation was assessed by measuring the incorporation of the [^{3}H]methyl moiety into phospholipids of cellular membranes. Pretreatment of NY945 (10 μg) significantly decreased histamine and leukotrienes releases during mast cell activation. The decrease of histamine release was stronger than that of leukotriene during mast cell activation. The phospholipase D activity increased by the mast cell activation was decreased by the dose-dependent manner in the pretreatment of NY945. The amount of DAG produced by PLC activity was decreased by NY945 pretreatment. The amount of mass 1, 2-diacylglycerol produced by activation of mast cells was decreased in the pretreatment of NY945. NY945 pretreatment strongly inhibited the incorporation of the [^{3}H]methyl moiety into phospholipids. The data suggest that NY945 purified from Aloe inhibits in part an increase of 1, 2-diacylglycerol which is produced by activating mast cells with antigen-antibody reactions, which is mediated via phosphatidylcholine-phospholipase D and phosphatidylinositol-phospholipase C systems, and then followed by the inhibition of histamine release. Furthermore, NY945 reduces the production of phosphatidylcholine by inhibiting the methyltransferase I and II, which decreases the conversion of phosphatidylcholine into arachidonic acid and inhibits the production of leukotrienes.

Key Words: Aloe glycoprotein, Mast cells, Histamine, Leukotrienes, Phospholipase D, 1, 2-diacylglycerol, Methyltransferase

Corresponding to: Jai Youl Ro, Department of Pharmacology, Yonsei University College of Medicine, CPO Box 8044, Seoul 120-752, Korea

INTRODUCTION

Medicines of plant-origin are commonly used in basic health care in many countries through the century. The preparations and uses of this type of medicines are largely based on empiricism. Despite the fact that a number of the preparations have been used successfully for ages, only little is known about the effects of plant constituents on the processes that cause the diseases.

A fairly well-documented preparation in traditional medicine is the whole or parenchymous leaf-gel of Aloe vera (Grindly & Reynolds, 1986). Aloe vera is one of the few substances known to effectively decrease inflammation and promote wound healing (David et al, 1989a; David et al, 1989b; David et al, 1991a; Shelton, 1991). Aloe vera is a complex plant containing many biologically active substances (Klein & Penneys, 1988). It has been reported that glycoprotein extracted by Aloe vera has the strong anti-inflammatory response (Davis, 1988; Davis et al, 1991b; Davis et al, 1992), and that polysaccharides, especially mannose-6-phosphate, in Aloe vera have the strong wound healing activity and anti-inflammatory response (Davis et al, 1994a). It has also been reported that sterols extracted from Aloe vera have good anti-inflammatory activity (Davis et al, 1994b).

Mast cells and basophils play a pivotal role in the pathogenesis of allergic reactions such as asthma. These reactions are a consequence of the release of granular mediators(histamine and 5-hydroxytryptamine etc.), newly synthesized mediators (leukotrienes, prostaglandins, and platelet-activating factor etc.), and cytokines such as interleukins (Plaut et al, 1989) and tumor necrosis factor (Ohno et al, 1990). When these cells are activated, the initial event in degranulation is the cross-linking of receptor-bound IgE antibody by a specific antigen, followed by the activation of tyrosine kinase (Kawakawa et al, 1992; Jouvin et al, 1994; Blank et al, 1995), phospholipase C (PLC) (Berridge & Irvine, 1984; Wolf et al, 1985; White et al, 1985; Berridge, 1987), phospholipase D (PLD) (Gruchalla et al, 1990; Lin et al, 1991; Lin et al, 1992; Stadelmann et al, 1993; Xie and Low, 1994; Rose et al, 1995), methyltransferase I and II (MT I and II)(Hirata et al, 1978; Hirata et al, 1979; Hirata & Axelord, 1980; Ishizaka et al, 1980; Daeron et al, 1982; Beaven et al, 1984; Beaven & Cunha-Melo, 1988; Takei et al, 1990; Ro & Kim, 1995), adenylate cyclase (Hirata et al, 1979), phospholipase A_2 (PLA_2) (Daeron et al, 1982), etc. The activation of these enzymes, especially PLC or PLD activation, ultimately lead to produce the 2nd messenger such as DAG which is known to be produced by tyrosine kinase activation in a rat mast cell line (Lin et al, 1994).

As described above, it can be inferred that effect of Aloe extracts on inflammation may have therapeutic relevance to allergic hypersensitivity and asthmatic disorders. Therefore, we first attempted to examine effects of crude Aloe vera extracts on the active systemic anaphylaxis, and then examined whether the isolated constituent of the Aloe vera, NY945, inhibits the mediator releases from guinea pig lung mast cells activated by specific antigen -antibody reactions. We also examined the mechanism of constituent of Aloe vera (NY945) on the mediator release during the mast cell activation.

METHODS

Animal

Hartley albino female guinea pigs, weighing about 200~250 g, were used. Animals were maintained along with "Principles of Laboratory Animal Care".

Drugs and solutions

Whole fresh leaf of Aloe vera was supplied from Nam Yang Corp (Seoul, Korea). The following substances were used: ovalbumin (OA), collagenase (type I), elastase (type I, porcine pancreatic), phosphatidylcholine (PC), lyso-PC (Sigma Chemical Co., Phillipsberg, NY); gelatin (Difco Laboratories, Ditroit, MI); percoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden); Polygram silica G TLC (Brinkmann Instruments Co, Westbury, NY); LK5DF and LK6D silica gel plates (Whatman, Maistone, Kent); [9, 10 (n)^{3}H]pahnitic acid (s.a., 53.0 Ci/mmol), [^{3}H] myristic acid (s.a., 51 Ci/mmol), L-[^{3}H-mehtyl] methionine (s.a., 83Ci/mmol), LTD_4 assay kit, (Amersham, Korea); phosphatidylethanol (PEt), phosphatidylbutanol (PBut), phosphatidylmonomethyl-ethanolamine (PMME), phosphatidyldimethylethanolamine (PDME) (Avanti Polar Lipids, Inc., Albaster, Ala). Several chemicals used in these studies and other reagents were of best grade.

Purification of Aloe vera

Fresh Aloe vera leaves (10 kg) were crushed with a commercial blender (LG electics co,. Korea) in 1/3 volumes of extraction buffers (50 mM sodium phosphate pH 8.0, 1.44 mM 2-mercaptoethanol, 1% polyvinylpyrolidine, 1 mM EDTA) and the slurries were collected. The slurries were filtered through cheese cloth and clarified by centrifugation at 10,000 $\times$g for 30 min at 4^{0}C. The supernatant of the crude extracts were precipitated with 25~80% ammonium sulfate saturation, and centrifuged as above. The precipitate was dissolved in 20 mM Tris-Cl (pH 8.0) buffer and was then desalted by application to a Sephadex G-25 column (8.0$\times$80 cm) equilibrated with the same buffer (W1P, crude extract used to the active systemic anaphylaxis).

The desalted extracts were applied to a DEAE-Sephacel column (3.4$\times$30 cm) equilibrated with 20 mM Tris-Cl (pH 8.0) buffer. The column was washed with the equilibration buffer. Bound proteins were eluted with 2 M NaCl in the same buffer. Fractions containing eluted protein were pooled, dialyzed, adjusted to 0.5 M NaCl in 20 mM Tris-Cl (pH 7.4).

The eluted proteins were chromatographyed through a column of Con A-Sepharose (3.4$\times$26 cm) equilibrated and washed with 20 mM Tris-Cl (pH 7.4) buffer containing 0.5 M NaCl. The elutes (negative charged proteins in the pH 8.0) were recovered in the pass through fraction and concentrated by ultrafiltration, dialysed 20 mM Tris-Cl (pH 7.4) buffer. Bound glycoproteins were eluted with a 0.5 M methyl-α-D-mannopyranoside in the same buffer, dialyzed against 20 mM Tris-Cl (pH 7.4) buffer. It referred to NY945.

Active systemic anaphylaxis (ASA)

48 BALb/c mice were grouped with 6 groups of 8 mice. The first group was injected by 0.9% saline as negative control group, 2nd group by the antigen (OA) adsorbed with $Al(OH)_3$ (OA-$Al(OH)_3$) alone as positive control group, 3rd group by 1 mg/kg Aloe (W1P, crude extract), 4th group by 2 mg/kg Aloe, 5 th group by 3 mg/kg Aloe, 6th group by 5 mg/kg Aloe. The first and 2nd groups were injected by i.p. with 0.2 ml saline, and 3rd to 6th groups were injected with each concentration of Aloe via i.p. one time every week for 4 wks. All the groups except the first group were injected by i.p. with 100 μg/0.2 ml OA-$Al(OH)_3$ in 5 wks. 2 wks after final injection, all mice were injected by 100 μ/0.2 ml OA-$Al(OH)_3$ via tail vein. We observed whether OA-induced anaphylatic shock occurs or not. The symptoms observed by the frequency of nasal rubbing and sneezing for 30 min were evaluated by 5 steps according to no symptoms (−), less than 10 times (±), more than 10 times (+), continuously (++), and death (+++).

Active sensitization protocol (anti-OA production)

Ten outbred female guinea pigs were first immunized by foot pad injections of mixture of 50 μg OA and complete freund's adjuvant. One week after that, animals received intradermal injections of 100 μg OA at one side back and 200 μg of OA at the other side back. Animals were sacrificed one week later and the sera were stored in aliquots at −70°C until the time of use. The quantity of serum antibody titers by passive cutaneous anaphylaxis (PCA) was determined as described in the previous article (Aderson, 1980). Serum IgG_1 antibody was separated by affinity column chromatography. Guinea pig blood serum was applied to anti-IgG_2 affinity column and 0.1 M citric acid (pH 2.1) was used to wash the column. IgG_1 was passed through and the absorbed IgG_2 antibody was rinsed by 0.2 M sodium carbonate (pH 11.3). The separated IgG_1 was concentrated under pressure for the experiment. The titers of anti-OA were 1,600~3,200. The sera were used for the preparation of passively sensitized mast cells.

Guinea pig lung mast cell preparations

Guinea pig lung mast cells were isolated and purified using techniques similar to the method previously reported (Undem et al, 1985). Briefly described here, lungs obtained from 16 unsensitized guinea pigs were each perfused with 50ml of the modified Tyrode buffer (TGCM) consisting of (millimolar): NaCl, 137; NaH_2PO4, 0.36; KCl, 2.6; $CaCl_2$, 1; $MgCl_2$, 1.5; $NaHCO_3$, 119; glucose, 5.5; gelatin, 1 g/L, pH 7.4. After removing large airways and blood vessels, the lungs were minced with a Mcllwain tissue chopper (The Mickle Laboratory Engineering Co. LTD, Gomshall, England). Pooled tissue was treated three times with 125 U/g tissue and 5 U/g tissue of collagenase and elastase, respectively. Times (min) of each consecutive exposure of lung fragments to the enzymes were 15, 15 and 25,

respectively. Freed cells were separated from residual tissue by filtration through mesh and Nytex mesh (100 μm). The resulting cell population was washed with Tyrode buffer without $CaCl_2$ and $MgCl_2$ containing gelatin (TG buffer) and layered over gradients consisting of 10 ml of percoll (density, 1.045/ml), and centrifuged at $800 \times g$ for 20 min. Pelleted cells (containing mast cells) were resuspended in TG buffer, and applied for further purification utilizing a discontinuous percoll density gradient (consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10 g/ml). This gradient was centrifuged at 800 $\times$g for 20 min ($\sim 3.5 \times 10^8$ cells/gradient). The cell band obtained between the 1.09 and 1.10 g/ml densities contained the highest purity and number ($1 \sim 2 \times 10^8$) of mast cells. This gradient band was removed, washed with TGCM buffer, and designated with partially purified mast cell preparation. Mast cell counts were obtained using alcian blue staining and cell viability was determined using trypan blue exclusion. Cell viability was consistently greater than 98%. The purity range of partially purified mast cells was 70~80%.

Mediator release from mast cells

The partially purified mast cells were passively sensitized with anti-OA serum (1 ml/10^6 cells) for 45 min at 37°C in a shaking water bath. After this incubation period, the cells were washed, resuspended in TGCM buffer and challenged with 0.1 μg/ml of OA for 10 min. Polystyrene tubes were used for all cell incubations, and unless stated otherwise, each tube contained 4×10^5 mast cells suspended in 1 ml of TGCM buffer. The mediator release reaction was terminated by placing the tubes in an ice bath. Supernatants obtained after centrifugation were taken for determination of histamine and leukotrienes. In the supernatants for the measurement of leukotrienes, 0.1% gelatin (final concentration) was added because leukotrienes were decomposed in air. In experiments utilizing NY945 (10, 30, 50 μg/4×10^5 cells), cells were first incubated with anti-OA for 45 min at 37°C after prelabeling, and then cells were prenicubated with NY945 for 5 min before challenge of 0.1 μg/ml OA.

Histamine assay

Histamine was analyzed by the automated fluorometric method (with dialyzer) described by Siraganian (1974). The sensitivity of the assay is approximately 5 ng/ml of histamine. The amount of histamine released was expressed as the percentage of the total histamine present in unstimulated cells.

Leukotriene Radioimmunoassay (RIA)

The leukotrine content of each cell supernatants was determined by RIA as described previously (Aharany, 1983). The leukotriene antibody was diluted in buffered saline (5 mM MES, HEPES adjusted to pH 7.4 with 1 N NaOH) containing 0.1% gelatin. Each assay tube contained 100 μl of supernatant, antibody (50 μl of a 1:1000 dilution), and 50 μl of [^{3}H]Leukotriene D_4 (LTD_4, 2500 to 3000 cpm) in buffered saline. Incubations were for 2 h at 4°C and the reaction was terminated by addition of 0.5 ml dextran coated charcoal (200 mg charcoal and 20 mg dextran mixed with 100 ml buffered saline). Five min after incubation the mixture was centrifuged at $800 \times g$ at 4°C and 0.4 ml of the supernatant was added to Aquasol (NEN Research Products) for counting by liquid scintillation spectrometry (Packard, Model 3225). Standard curves were constructed in the presence of antigen using LTD_4. The detection limit of the assay is 0.045 pmole LTD_4 release in expressed as pmole/4×10^5 cells.

Prelabeling mast cells

In order to label mast cell phospholipids, purified cells were prelabeled with [^{3}H]palmitic acid (PlA). Purified cells ($1 \sim 2 \times 10^7$) were suspended in a final volume of lml TGCM and [^{3}H]PlA (at final concentration of 3.3 μM; 200 μCi/ml), and incubated at 37°C for 1 hr. Cells were washed twice and resuspended in TGCM before use in cell activation.

Sensitization, stimulation, and lipid extraction in mast cells

Prelabeled cells ($0.75 \sim 1.25 \times 10^6$) were sensitized by anti-OA antibody (IgG_1, 1 ml antibody/10^6 cells) at 37°C for 45 min, and washed and resuspended in TGCM. Prelabled and sensitized cells (1×10^6 cells) added with phosphatidylserine (PS, 15 μg/ml) were stimulated at 37°C for 10 min by specific antigen (0.1 μg/ml OA) or PS alone in a final 200 μl volume in 5 ml polypropylene tubes. Ethanol or butanol (50

mM) was added 5 min before stimulation. Reactions were stopped by adding 2 ml cold TGCM and centrifuged for 10min at 800×g. Cellular lipids were extracted from the cell pellet by using a modification (Gruchalla et al, 1990) of the Bligh and Dyer procedure (1959). In experiments utilizing NY945, cells were incubated with anti-OA after prelabeling, and then cells were preincubated with NY945 for 5 min before challenge of 0.1 μg/ml OA.

Separation of labeled phospholipids by TLC

A double one-dimensional TLC (Polygram precoated silica gel G plates, 10×20 cm) was used to separate phospholipids of interest from extracted lipids. A series of samples containing PEt were spotted 12 cm from the bottom of the plate and developed in hexane/ether/acetic acid (30:70:1) to 20 cm in order to resolve labeled neutral lipids from phospholipids that remained at the origin. Plates were cut at 0.8 cm above the origin, rotated 180°, and developed up to the top with chloroform/methanol /ammonium hydroxide (65 : 35 : 5). After they had dried, plates were sprayed with En^{3}Hance, and autoradiography performed by using Kodak XAR film for 1 week. Radioactive bands from the other plate were removed from the silica plates by gentle scraping, placed, and counted in scintillation vials containing 5 ml of scintillation cocktail. The identities of labeled band (PEt) on Rf values obtained for authentic neutral lipids and phospholipids visualized by iodine staining.

In the experimental mixture adding the butanol (50 mM), the cellular lipids were extracted after reaction stop from the cell pellet by using a modification of the Bligh and Dyer procedure (1959). The standards (phosphatidic acid and PBut) with extracted samples for the measurement of [^{3}H]PBut were applied to the oxalated-treated TLC (LK6D silica gel 60, Whatman) plates (presorbed-TLC, 5 g potassium oxalate dissolved in 250 ml H_2O, make up to 500 ml with methanol), developed to the top of the TLC plates with ethylacetate/acetic acid/2, 2, 4-trimethylpentane (9/2/5), and visualized standards with iodine staining. PA had an Rf value of 0.46, and that for PBut was 0.81. TLC plates were scraped, and counted. Butanol as the alcohol of choice for this experiments was used because of the fivefold lower potency of ethanol as an acceptor in the transphosphatidylation reaction.

The standard (DAG) with the extracted samples for the measurement of [^{3}H]DAG by using 100 mM butanol were applied to the presorbed-TLC plates and developed by diethyl ether/hexane/acetic acid (70/30/1). The only complication for the DAG assays based on labeling of phospholipids applied in this way is that it is necessary that phosphatidylalcohols as well as PA do not interfere in the separation system used. Both PA and PBut in the these sample mixture remain at the same place applied without migration. An Rf value for DAG was 0.71.

Measurement of mass 1, 2-diacylglycerol during mast cell activation

Prelabeled cells ($1\sim1.25\times10^6$) with [^{3}H]myristic acid (1 M, 1 Ci) were sensitized by anti-OA (1 ml/10^6 cells) at 37°C for 45 min and stimulated by 0.1 μg/ml OA at 37°C for time the indicated as described (Lin et al, 1994). The reactions were stopped by adding 1 ml of methanol. The labeled lipids were extracted by Bligh and Dyer method (1959). The standard with extracted samples was applied to the presorbed-TLC plates, and developed up to the half of the TLC plates with ethylacetate /acetic acid/ triethylpentane (9/2/5). The TLC plates after air dry were run up to the top of the TLC plates in a second system with hexane/diethyl ether/methanol/acetic acid (90/20/3/2). The location of [^{3}H] DAG was checked by exposure to iodine vapour. The TLC plates were scraped to measure radioactivity. NY945 was added during all procedures to NY945 groups. The Rf value for DAG after prelabeling with [^{3}H]myristic acid were 0.55.

Determination of phospholipid methylation during mast cell activation

Phospholipid methylation was determined as described by Ishizaka et al (1980). The purified mast cells sensitized with anti-OA (1×10^6 cells/ml) were incubated (25 min, 37°C) with L-[^{3}H-methyl] methionine (2 μM, 165 μCi/ml), and washed twice. The resuspended cells were challenged with 0.1 μg/10 μl OA for the stated times. The reaction was stopped by the addition of 900 μl of ice-cooled 10% TCA containing 10 mM L-methionine, and tubes were centrifuged at 1,000×g for 10 min at 4°C. The precipitates were washed with 10% TCA and then extracted with 3 ml of chloroform/methanol (2:1 v/v).

The chloroform phase was washed twice with 1.5 ml of 0.1 M KCl in 50% methanol. A 1 ml fraction of the chloroform phase was transferred to a counting vial and evaporated to dryness at room temperature. The residue was counted.

Identification of methylated phospholipid was carried out by TLC as described by Hirata et al (1980). The chloroform layer obtained from the chloroform/methanol extracts were evaporated under the N_2 gas, and the residue was dissolved in 50 μl of chloroform/methanol (2:1 v/v). Then 30 μl of each solution was applied to a TLC plate (LK5DF, Whatman), and developed for 4 hr with n-propanol /propionic acid/chloroform/water (3:2:2:1 v/v). Lipid spots identified with iodine staining and corresponding to known Rf values of authentic standards (PMMC, PDME, PC and Lyso-PC) were scraped into scintillation vials, and dispersed by sonication with 400 μl of methanol and 5 ml of scitillation solution, and radioactivity was counted.

Statistic analysis

Experimental data were shown as mean S.E.M.S. An analysis of variance (ANOVA) was used for statistical analysis. An analysis of significance between each control group and experimental group was carried out with the Scheffe method. When P values were less than 0.05 or 0.01, it was considered significant.

RESULTS

The effect of crude Aloe extract (W1P) on active systemic anaphylaxis

Given the fact that crude Aloe extract was effective on the anti-inlammation from the previous studies, we hypothesized and examined if crude Aloe extract can affect antigen-induced anaphylatic shock. The result showed that all mice of positive control group injected with 100 μg/0.2 ml of OA-Al(OH)$_3$ strongly had the symptoms of anaphylactic shock and finally died. When pretreated with 1 mg/kg or 2 mg/kg of W1P for 4 wks, the moderate anaphylactic shock (+) was observed in 3 mice out of 8 and 1 out of 8 mice, respectively. When pretreated with more than 3 mg/kg of W1P, any symptoms of anaphylactic shock was not observed (Table 1). Therefore, we made an attempt of purification of Aloe vera the same as described in "Materials and Methods". NY945 yielded by the various purification process was used in this study.

The effect of NY945 on the mediator releases during mast cell activation

In order to examine whether NY945 itself has the mediator releasing effect on hypersensitivity reactions, unsensitized mast cells were challenged by OA, 0.1 μg/ml, after the pretreatment of the varying concentrations of NY945 or mast cells sensitized with

Table 1. Experimental design of active systemic anaphylaxis, and symptoms of BALb/c mice after OA-Al(OH)$_3$ challenge[a]

Group	Pre-treatment	Sensitizing antigen	Challenging antigen	Severing of anaphylaxis				
					±	+	++	+++
1	Saline	Saline	Saline	8[b]	–	–	–	–
2	Saline	OA-Al(OH)$_3$ (100 μg/0.2 ml)	OA-Al(OH)$_3$ (100 μg/0.2 ml)	–	–	–	–	8
3	Aloe 1 mg/kg	"	"	1	4	3	–	–
4	2 mg/kg	"	"	4	3	1	–	–
5	3 mg/kg	"	"	8				
6	5 mg/kg	"	"	8				

[a]. Each group was injected by ip and iv following experimental design for 7 wks, and observed the degree of symptoms, the same as described in "Material and Method".
[b]. Number of animal in each group was 8 mice.

Table 2. Effect of NY945 on the antigen-induced releases of histamine and leukotrienes in guinea pig lung mast cells sensitized with antibody[a]

Treatment	Histamine(%)	Leukotrienes (pmoke/4×10^5 cells)
OA alone	27.9±2.19	15.4±2.17
NY945		
10 μg	8.5±2.09**	11.2±1.20*
30 μg	5.7±1.87***	9.6±0.98**
50 μg	1.1±1.01***	8.2±1.23**

[a]. Guinea pig lung mast cells were isolated and purified by digestion and rough and continuous percoll density gradient method. Mast cell(4×10^5 cells) were passively sensitized by anti-OA antibody and challenged by OA, 0.1 μg/ml. *, P<0.05, **, P<0.01, *** P<0.001 by comparison with OA alone.

anti-OA antibody were challenged by varying concentrations of NY945 (1, 5, 10, 30, 50, 100 μg/ml). The results showed that NY945 itself did not affect the mediator releases (data not shown).

We examined the effect of NY945 on the histamine and leukotriene releases from guinea pig lung mast cells activated on OA-anti-OA antibody complexes. When the mast cells sensitized with anti-OA antibody were challenged by 0.1 μg/ml OA after pretreatment of NY945, 10 μg/ml, histamine release was 8.5±2.09% and that showed a 69.5% decrease when compared to the OA alone which was 27.9±2.19%. The amount of leukotriene released by 10 μg/ml NY945 pretreatment was 11.2±1.20 pmole/4×10^5 cells, which was a 27.3% decrease when compared to the 15.4±2.17 pmole/4×10^5 cells of OA alone group (Table 2). The inhibitory effect of both mediator releases by NY945 pretreatment showed the dose-dependent manner. In this study 50 μg of concentration of NY945, which decreased up to the maximum both mediator released from activated mast cells, was mainly used in each experiment.

The effect of NY945 on the activation of the phospholipase D in mast cells activation

An increase of membrous PLD activity during mast cell activation evoked by specific antigen-antibody reactions ultimately leads to release the mediators from mast cells. Therefore, the effects of NY945 on increasing PLD activity in mast cells activated by OA-anti-OA antibody reactions were studied. PLD activity was measured that in the presence of ethanol or butanol, phospholipid changed

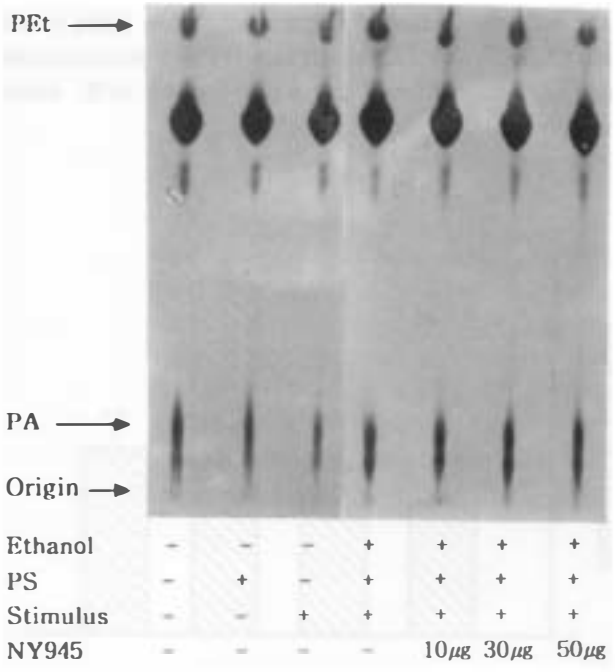

Fig. 1. Autoradiogram of ethanol-dependent phosphatidylethanol formation in the activated mast cells after NY945 pretreatment. Purified mast cells ($1\sim2\times10^7$) were prelabeled with [^{3}H]palmitic acid (0.8 μM, 50μCi), and washed extensively. The prelabeled cells were sensitized with anti-OA antibody (1 ml/10^6 cells) in the absence or presence of NY945 for 45 min. Five min before OA (0.1 μg/ml) challenge, 0.5% ethanol was added and challenged by OA for 10 min. The PEt was determined by Materials and Methods described above. The abbreviation used are: PA, phosphatidic acid; PS, phosphatidylserine. The data is shown in the results.

into transphosphatidylation reaction involving the transfer of the phosphatidyl moiety of the phospholipid substrate to ethanol or butanol thereby producing phosphatidylethanol (PEt) or phosphatidylbutanol (PBut). As shown in Fig. 1, in the mast cells sensitized only with 0.1 μg/ml of OA, the PEt production, which is the index of the measurement of PLD activity, increased remarkably, and phosphatidic acid (PA) production decreased. PLD activity increased more than 2 times in the mast cells activated by antigen challenge (from 1856±148 cpm to 4419±241 cpm). However, the production of PEt was significantly decreased from 4419±241 cpm to 2382±301 cpm after NY945 (50 μg) pretreatment (Fig. 1). The production of PBut in activated mast cell increased remarkably from 2114±56 cpm to 5564±146 cpm but with NY945 (50 μg) pretreatment the PBut was deceased remarkably from 5564±149 cpm to 2907±299 cpm. In the NY945 pretreatment the PLD activity was decreased up to 48% when compared to antigen alone (Fig. 2).

The effect of NY945 on the production of 1, 2-diacylglycerol during mast cell activation

The production of DAG, which is a 2nd messenger strongly related with histamine release during mast cell activation, is already known to have biphasic nature such as initial increase of DAG production at 5~10 sec and prolonged increase of DAG for more than 30 min during mast cell activation. Since NY945 strongly reduced the increasing activity of PLD in activated mast cells with specific antigen-antibody reactions, it can be inferred that NY945 decreases the production of DAG by inhibiting the PLD activity, which is known to induce the production of DAG by indirect pathway system. Therefore, we examined the effect of NY945 on the production of DAG caused by mast cell activation. When the mast cells (1×10^6 cells) were labeled with [^{3}H]palmitic acid, sensitized with anti-OA and challenged with 0.1 μg/ml OA for 10 min in the presence of high concentration of butanol (100 mM), the amounts of DAG, which

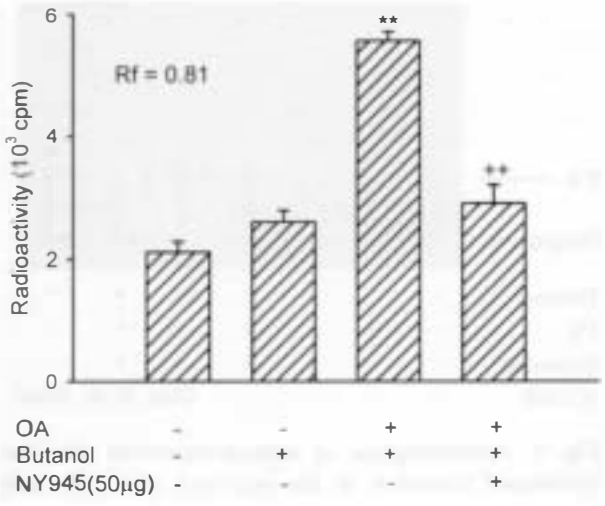

Fig. 2. Butanol-dependent PBut formation in the activation of purified guinea pig lung mast cells sensitized with anti-OA. Purified mast cells ($1\sim2\times10^7$) were prelabeled with [^{3}H]palmitic acid (0.8 μM, 50 μCi), and washed extensively. Cells were sensitized with anti-OA antibody (1 ml/10^6 cells) for 45 min. Five minutes before OA (0.1 μg/ml) challenge, 50 mM butanol and NY945 (50 μg) were added and challenged by OA for 10 min. The PBut was determined by Materials and Methods described above. **, P, 0.01 by comparison without OA challenge. ++, P<0.01 by comparison with OA challenge in the presence of butanol(50 mM).

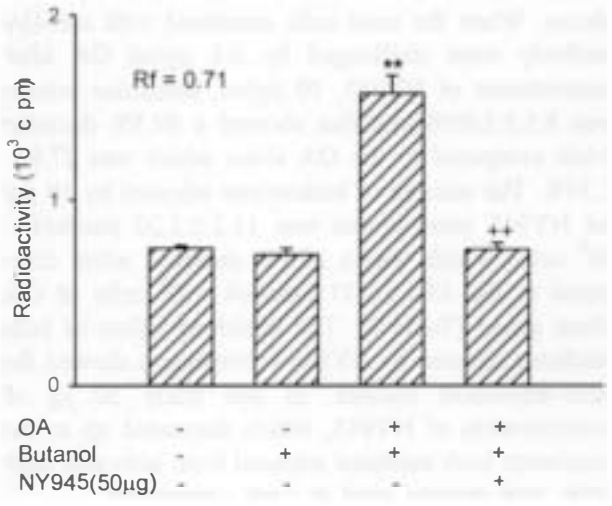

Fig. 3. Effects of NY945 on the DAG formation in the presence of butanol during the activation of mast cells sensitized with anti-OA. Purified mast cells ($1\sim2\times10^7$) were prelabeled with [^{3}H]palmitic acid(0.8 μM, 50 μCi), and washed extensively. Cells were sensitized with anti-OA antibody (1 ml/10^6 cells) for 45 min. Five minutes before OA(0.1 μg/ml) challenge, 100 mM butanol and NY945(50 μg) were added and challenged by OA for 10 min. [^{3}H]DAG was determined by Materials and Methods described above. **, P<0.01 by comparison without OA challenge. ++, P<0.01 by comparison with OA challenge in the presence of butanol (100 mM).

means the amounts of DAG produced by PI-PLC pathway, were 15787±951 cpm. In the NY945 (50 μg/ml) pretreatment the amounts of DAG were 7297 ±354 cpm. These results showed 53.7% decrease when compared to antigen alone (15787±951 cpm)(Fig. 3).

The effect of NY945 on the production of mass 1, 2-diacylglycerol during mast cell activation

As seen above, the production of DAG was decreased, when pretreated with NY945 during mast cell activation. Therefore, the effect of this phenomenom on the biphasic nature of DAG production was checked. The mast cells stimulated by antigen-antibody reaction show a biphasic nature, the early increase (30～60 sec) and continual increase (1～30 min), and this was also reconfirmed in this study. In short, when the mast cells (1×10^6 cells) sensitized with anti-OA were labeled with [^{3}H] myristic acid, and challenged with 0.1 μg/ml OA for times specified, the production of mass DAG reached its climax at 30～60 sec, and there is a decrease of DAG production at 1～2 min and this continues for 30 min (Fig. 4 showed up to 10 min although observation continued for 30 min). The amount of initial DAG (30 sec) was 14214±958 cpm, secondary increase of DAG (10 min) was 11218±859 cpm. Both DAG production was over 2.0～2.5 times, compared with the non-antigen stimulated mast cells (5585±371 cpm). The pretreatment of NY945 (50 μg) decreased up to 35.9% (initial DAG production) and 50.6% (continuous DAG production), respectively (from 14214±958 cpm to 9111±389 cpm and from 11518 ±859 to 6704±545 cpm).

The effect of NY945 on methylation of lipids during mast cell activation

The effect of NY945 on phospholipid methylation activity in guinea pig lung mast cells was examined

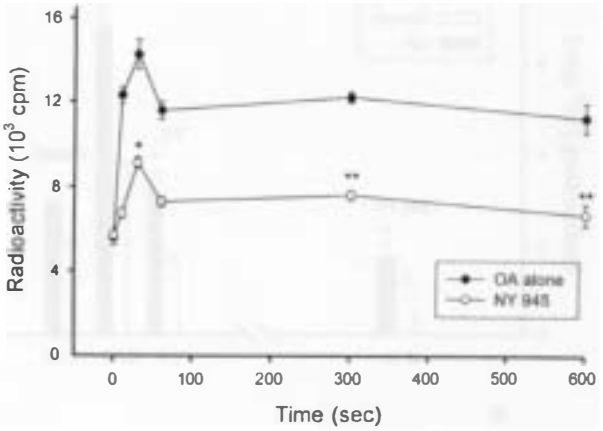

Fig. 4. Effects of NY945 on the mass DAG formation during the activation of mast cells sensitized with anti-OA. Purified mast cells (1×10^6) were labeled with [^{3}H]myristic acid (1 μM, 1 μCi) for 1hr, sensitized with anti-OA antibody (1 ml/10^6 cells) for 45 min, and then challenged with OA (0.1 μg/ml) for times specified after pretreatment of NY945 (50 μg). [^{3}H]DAG was extracted and separated from other lipids as described in the Methods. *, $P<0.01$; **, $P<0.001$ by comparison with OA alone.

Table 3. Effect of NY945 on the [^{3}H]methyl moiety incorporation and leukotriene release caused by OA in guinea pig lung mast cells sensitized with anti-OA

Treatment	Phospholipid methylation (CPM)[a]	Leukotriene release (pmole/4×10^5 cells)
OA alone	17450±576[b]	15.4±2.17
NY945		
10 μg	13585±773	11.2±1.20*
30 μg	11653±441**	9.6±098**
50 μg	9772±359**	8.2±1.23**

[a]. Guinea pig lung mast cells (1×10^6 cells) were passively sensitized by anti-OA, prelabeled by L-[^{3}H]methyl methionine, and challenged with OA at 5 min after each NY945 pretreatment.
[b]. Valuea represent maximum methylation of phospholipids at 15 sec after challenge of OA (0.1 μg/4×10^5 cells).
*. $P<0.05$, ** $P<0.01$ by comparison with OA alone.

after the addition of an optimal concentration of OA (0.1 $\mu g/4 \times 10^5$ cells).

It is previously reported that the incorporation of the [^{3}H]methyl moiety into the phospholipids reached a maximum at 15 sec after the addition of OA, 0.1 $\mu g/4 \times 10^5$ cells (Takei et al, 1990). The incorporation of the [^{3}H]methyl moiety into the membrane phospholipids in cells pretreated with NY945 (50 $\mu g/1 \times 10^6$ cells) was inhibited markedly (Table 3). The inhibition of methylation with NY945 (50 μg) was 43.5% at 15sec after challenging of OA (from 17450 $\pm$375 cpm to 9772$\pm$359 cpm).

We also examined each methylated products during the activation of mast cells by thin-layer chromatography. The results of maximal phospholipid methylation after challenging of OA (15 sec) showed in Fig 5. As observed in the stimulated control(Fig 5a), the [^{3}H]methyl moiety was obviously incorporated into phospholipids of mast cells activated with antigen-antibody reactions (OA-anti-OA). The incorporated amount of [^{3}H]methyl moiety in each lipid product markedly increased in the mast cells activated with antigen, compared to the unstimulated control (Fig. 5a) (Lyso-PC, from 1161$\pm$160 cpm to 1748$\pm$324 cpm; PC, from 1672$\pm$89 cpm to 3205$\pm$463 cpm; PDME, from 3853$\pm$230 cpm to 6766$\pm$439 cpm; PMME, from 2048$\pm$113 cpm to 2974$\pm$92 cpm). NY945 strongly inhibited each [^{3}H]methyl incorporation into phospholipid(Fig. 5b) (Lyso-PC, from 1748$\pm$324 cpm to 1189$\pm$272 cpm; PC, from 3205$\pm$463 cpm to 1667$\pm$314 cpm; PDME, from 6766$\pm$439 cpm to 3929$\pm$389 cpm; PMME, from 2974$\pm$92 cpm to 1843$\pm$271 cpm).

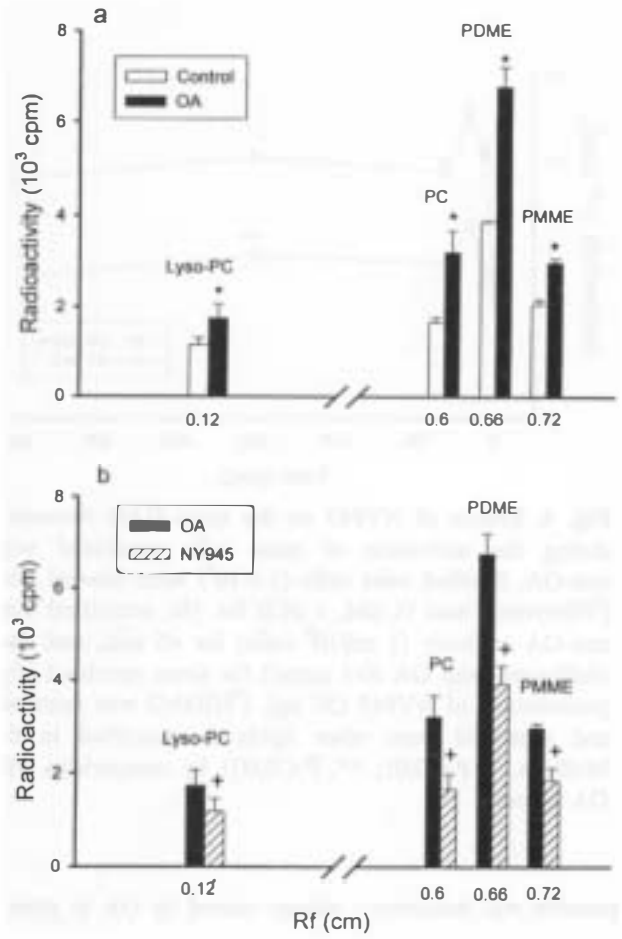

Fig. 5. Chromatographic pattern of each [^{3}H]methylated phospholipids on a TLC plate in the absence or presence of NY945. Purified mast cells (1×10^6) were sensitized with anti-OA antibody (1 ml/10^6 cells), labeled with [^{3}H-methyl]methionine (2 μM, 165 μCi), and then challenged with OA(0.1 μg/ml) for 15 sec at 37°C. The incorporation of methyl moeiety of each phospholipids was separated by TLC as described in the methods. (a), unstimulated vs stimulated mast cells; (b), pretreated with NY945 (50 $\mu g/1 \times 10^6$ cells). Blank column, non-antigen challenge; black column, antigen challenge; hatchered column, NY945 pretreatments. Values are means$\pm$SE for experiments. *, $P<0.05$ by comparison with OA challenge. +, $P<0.05$ by comparison with OA alone.

DISCUSSION

Aloe contains a variety of substances and factors which modulate the immune system. It has been reported that Aloe vera has a stimulatory system which enhances antibody production (Hart et al, 1988; Davis et al, 1989b; Davis et al, 1991a; Sheton, 1991), and an inhibitory system that influences both inflammatory and immune responses (Davis et al, 1991b; Davis et al, 1992; Davis et al, 1994a). The interaction between these two systems is referred to as biological modulation. In this study we focused on inhibitory system of Aloe vera.

First of all we examined the effect of crude extract (W1P) of aloe vera on the active systemic anaphylaxis. The symptoms of active systemic anaphylaxis in animal pretreated with more than 3 mg/kg W1P were not observed (Table 1). These results can infer that Aloe vera contains some inhibitory components acting in allergic hypersensitivity reactivity. Therefore, some inhibitory components in Aloe vera were purified by a various of purification process. We

yielded NY945 which is separated to the three band by SDS-PAGE and supposed to be glycoprotein (see the purification method). Each band of NY945 and NY945 itself showed similar effect each other in allergic hypersensitivity reactivity. Therefore, we are continuously doing the purification of NY945, and we here report results yielded from NY945.

In this experiment, we observed that NY945 strongly inhibits histamine and leukotriene releases during the activation of mast cells by specific antigen-antibody complexes (Table 2). The inhibitory effects of mediator releases by NY945 agree with several reports that extracts of Aloe vera has anti-inflammatory activity in the synovial pouch model and the chemicals-induced edema (Davis et al, 1991b; Davis et al, 1992; Davis et al, 1994a; Davis et al, 1994b).

Since the inhibition of histamine release by NY945 was stronger than that of leukotriene release, we first attempted to confirm the inhibitory mechanism of NY945 on the histamine release caused by mast cells activated with specific antigen-antibody complexes. When mast cell membrane receptors are activated by antigen-antibody complexes, the enzyme system in the cell membrane are activated. The activated enzymes (PLC, PLD, PLA_2, adenylate cyclase, MT I and II etc) are intimately related to the generation of a number of second messengers (inositol triphosphates, IP_3 and DAG etc). The results lead to exocytosis of performed inflammatory mediators and synthesis of newly formed mediators and then can induce asthma and allergic hypersensitivity.

The most common secondary messenger which is related with histamine release is DAG. The DAG can be formed from PC either indirectly by a PLD -initiated pathway or from other phospholipids directly by the activation of PLC with receptor-mediated cell activation (Berridge & Irvine, 1984; Wolf et al, 1985; Gruchalla et al, 1990; Lin et al, 1992). Recently, it has been reported that the amount of DAG produced by PLD activity during the activation of rat peritoneal mast cells was greater than that by PLC activity (Gruchalla et al, 1990; Andrew et al, 1996). This study first focused on PLD activity to examine the inhibitory mechanism of NY945 on the histamine release. It has previously been reported that the production of PEt or PBut, which is an index for measuring PLD activity in the presence of ethanol or butanol, increased 2～3 times during guinea pig lung mast cell activation (Ro & Kim, 1995). This increased production of PEt or PBut evoked during mast cell activation is decreased by NY945 pretreatment (Fig. 1～2). Therefore, these results can be indirectly inferred that NY945 inhibits PLD activity during the activation of mast cells sensitized with specific antigen-antibody reactions.

Since the inhibition of PLD activity by the pretreatment of NY945 was observed, the effects of NY945 on the indirect DAG production from phospholipids by phosphatidic acid phosphohydrolase (PA Phase) enzyme activity via PLD activation and on the direct DAG production by action of PLC during antigen-antibody reactions in mast cells was examined. DAG is a secondary messenger related to the histamine release caused by the stimulated mast cells. There are many reported methods to measure for DAG (Lee et al, 1991; Huang et al, 1992; Lin et al, 1994). First, when mast cells are stimulated with antigen-antibody reactions the DAG produced by PLD activity is totally turned into PBut under the influence of a high concentration of butanol (100 mM). Then the only DAG amount directly produced by PLC activity can be measured. NY945 pretreatment reduced the increased DAG produced when challenged by antigen alone (Fig. 3). These results show that NY945 inhibits DAG production via PLC activity stimulated by mast cell activation.

The effect of NY945 on mass DAG production through the actions of PLC and PLD was also observed. The pathway of mass DAG production was composed of biphasic nature in cultured cells. That is, the increase of DAG production in the early stages was caused by other phospholipase (ex. PLC) and its continual increase by tyrosine kinase activity causing activation of PLD (Lin et al, 1992; Jouvin et al, 1994; Blank et al, 1995) were checked in the mast cells of guinea pig lung tissues. The mass DAG produced by the mast cell activation of guinea pig lung was found to have biphasic nature (Fig. 4), and the results are the same as the report that IgE-dependent DAG production in RBL 2H3 cultured cells have a biphasic nature (Lin et al, 1992). Therefore, it can be inferred that the nature of DAG production, which is related with histamine release caused by immune reaction, is the same regardless of the distributions of tissues or species. When NY945 is pretreated, both DAG amounts produced from early stage caused by PLC activity and continual increase caused by PLD activity are blocked (Fig. 4). From these results it can be inferred that NY945 inhibits DAG production via

both PI-PLC and PC-PLD pathways. As a result, histamine release is reduced.

The effect of NY945 on phospholipid methylation activity in guinea pig lung mast cells was also examined after the challenge of OA. The degradation products of the methylated phospholipids such as PMME, PDME, PC and LPC was identified by thin-layer chromatography. Based on the experimental protocol of Ishizaka et al (1980), we reconfirmed a transient increase in [^{3}H]methyl incorporation at 15sec from OA-activated mast cells. NY945 remarkedly inhibited the incorporation of [^{3}H]methyl moiety into phospholipid (Table 3). The data obtained with TLC infer that NY945 may inhibit both MT I and II, and phospholipase A_2 (PC hydrolysis to arachidonic acid and Lyso-PC). Therefore, these results show that NY945 inhibits the phospholipid methylation (especially PC product) induced by antigen, followed by inhibition of leukotriene release.

From these results, it can be inferred that NY945 inhibits histamine release by inhibiting DAG production from phospholipids during mast cell activation, which is mediated via phosphoinositide- PLC and phosphatidylcholine-PLD systems. Furthermore, NY945 reduces the phosphatidylcholine production by inhibiting the methyltransferase, which decreases the conversion of phosphatidylcholine into arachidonic acid and inhibits the production of leukotrienes.

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