The Inhibitory Mechanism of Aloe Component (NY945) on the Mediator Releases evoked with Mast Cell Activation

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ABSTRACT

By using guinea pig lung mast cells, this study aimed to examine the effects of Aloe component(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 cells 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 more directly by the production of labeled phosphatidylethanol or phosphatidylbutanol which was produced by phospholipase D-mediated transphosphatidylation in the presence of ethanol or butanol. The amount of mass 1,2-diacylglycerol was measured by the [^{3}H]1,2-diacylgycerol produced when prelabeled with [^{3}H]myristic acid. In the mast cells prelabeled with L-[^{3}H]methyl methionine the phospholipid methylation was assessed by measuring the incorporation of the [^{3}H]methyl moiety into phospholipids. Pretreatment of NY945(10μg) significantly decreased histamine and leukotrienes releases during mast cell activation. The decrease of histamine release was stronger than that of leukotrienes 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 mass 1,2-diacylglycerol produced by activation of mast cells were 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 complexes which is mediated via phosphatidylcholine-phospholipise D and phosphatidylinositole-phospholipise C systems, and then followed by the inhibition of histamine release. Furthermore, NY945 reduces the phosphatidylcholine production by inhibiting the methyltransferase I and II, which decrease the conversion of phosphatidylcholine into arachidonic acid and inhibits the production of leukotrines.

INTRODUCTION

Aloe vera is one out of a fairly well-documented preparations in traditional medicines(1). Aloe vera also includes one of the few substances known to effectively decrease inflammation and promote wound healing(2-5). Aloe vera is a complex plant containing many biologically active substances(6). It has been reported that

glycoprotein extracted by Aloe vera has the strong anti-inflammatory response(7-9), and that polysaccharides, especially mannose-6-phosphate, in Aloe vera have the strong wound healing activity and anti-inflammatory response(10). It has also been reported that sterols extracted by Aloe vera have good anti-inflammatory activity(11).

Mast cells and basophils play a critical 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(12) and tumor necrosis factor(13). 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 phospholipase C(PLC)(14-17), phospholipase D(PLD)(18-20), methyltransferase I and II(MTI and II)(21-28), adenylate cyclase(22), phospholipase A_2(PLA_2)(24), etc. The activation of these enzymes, especially PLC or PLD activation, ultimately lead to produce the 2nd messenger such as 1,2-diacylglycerol(DAG) which is known to be produced by tyrosine kinase activation in a rat mast cell line(29). The activation of MT I and II enzymes in the membrous mast cells evoked by antigen-antibody complexes also causes a rise in Ca^{++} influx which increases the activity of PLA_2. The results lead to the hydrolysis of phosphatidylcholine(PC) with subsequent arachidonic acid and lyso-PC releases. This arachidonic acid is what makes leukotrienes(21-28).

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 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 releases during the mast cell activation.

MATERIALS AND METHODS

Animal

Hartley albino female guinea pigs, weighing about 200-350g, were used.

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), tyrphostin (Sigma Chemical Co., Phillipsberg, NY); gelatin(Difco Laboratories, Ditroit, MI); percoll(Pharmacia Fine Chemicals AB, Uppsala, Sweden); Polygram sil G TLC (Brinkmann Instruments Co, Westbury,NY); LK5DF and LK6D silica gel plates (Whatman, Maistone, Kent); [^{3}H]myristic acid (s.a., 51Ci/mmol), LTD_4 assay kit, (Amersham, Korea); phosphatidylethanol, phosphatidylbutanol(Avanti Porar Lipids). Several chemicals used in these studies and other reagents were of best grade.

Purification of Aloe vera

Fresh Aloe vera leaves (10kg) were crushed with a commercial blender (LG electics co,. Korea) in extraction buffers (50mM sodium phosphate pH 8.0, 1.44 mM

2-mercaptoethanol, 1% polyvinylpyrolidine, 1mM EDTA). The slurries were filtered through cheese cloth and clarified by centrifugation at 10,000 x g for 30min at 4^0C. The supernatant of the crude extracts were precipitated with 25-80% ammonium sulfate saturation. The precipitate was dissolved in 20mM Tris-Cl (pH 8.0) buffer and was then desalted by application to a Sephadex G-25 column (8.0 x 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 equilibrated with 20 mM Tris-Cl (pH 8.0) buffer. Bound proteins were eluted with 2 M NaCl in the same buffer. The eluted proteins were chromatographyed through a column of Con A-Sepharose equilibrated 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. 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 1mg/kg Aloe (W1P, crude extract), 4th group by 2 mg/kg Aloe, 5th group by 3 mg/kg Aloe, 6th group by 5 mg/kg Aloe. The first and 2nd groups were injected by i.p. with 0.2ml saline, and 3rd to 6th groups were injected with each concentration of Aloe via ip one time every week for 4 wks. All the groups except the first group were injected by i.p. with 100μg/0.2 ml OA-$Al(OH)_3$ in 5 wks. 2wks after final injection, all mice were injected by 100μg/0.2 ml OA-$Al(OH)_3$ via tail vein. We observed whether OA-induced anaphylatic shock occurs or not. The symptoms were evaluated by 5 steps according to no symptoms (-), less moderate ($\pm$), moderate(+), more moderate (++), and death (+++).

Active sensitization protocol (anti-OA production)

Ten outbred female guinea pigs were first immunized by foot pad injections of mixture of 50μg OA and complete freund's adjuvant. One week after that, animals received intradermal injections of 100μg OA at one side back and 200μg of OA at the other side back. Animals were sacrificed one week later and the sera were stored in aliquots at -70℃ until the time of use. The quantity of serum antibody titers by passive cutaneous anaphylaxis (PCA) was determined as described in the previous article(30). 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(31). The lungs(16 guinea pigs) were minced with a Mcllwain tissue chopper (The Mickle Laboratory Engineering Co. LTD, Gomshall, England). Pooled tissue was treated three times with 125U/g tissue and 5U/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. 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 10ml of percoll (density, 1.045/ml), and centrifuged at 800 x g for 20 min. Pelleted cells (containing mast cells) were applied for further purification utilizing a discontinuous percoll density gradient (consisting of densities 1.06, 1.07, 1.08, 1.09, and 1.10g/ml). This gradient was centrifuged at 800 x g for 20 min ($\sim 3.5x10^8$ cells/gradient). The cell band obtained between the 1.09 and 1.10g/ml densities contained the highest purity and number ($1-2x10^8$) of mast cells. This gradient band was removed, washed with TGCM buffer, and designated with partially purified mast cell preparation. 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 (1ml/10^6 cells) for 45 min at 37℃ in a shaking water bath and challenged with 0.1μg/ml of OA for 10 min. 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 experiments utilizing NY945 (10, 30, 50μg/$4x10^5$ cells), cells were first incubated for 45min at 37℃ concomitantly with anti-OA and NY945 prior to incubation with OA.

Histamine assay

Histamine was analyzed by the automated fluorometric method (with dialyzer) described by Siraganian(32). The sensitivity of the assay is approximately 5ng/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(33). The detection limit of the assay is 0.045 pmole LTD_4 release in expressed as pmole/$4x10^5$ cells.

Prelabeling, sensitization, stimulation, and lipid extraction in mast cells

In order to label mast cell phospholipids, purified cells were prelabeled with [^{3}H] palmitic acid(PlA). Purified cells($1-2 x 10^7$) were suspended in a final volume of 1ml TGCM and [^{3}H]PlA (at final concentration of 3.3μM; 200 μCi/ml), and incubated at 37℃ for 1hr. Prelabeled cells($0.75-1.25x10^6$) were sensitized by anti-OA (IgG_1, 1ml antibody/10^6 cells) antibody at 37℃ for 45 min. Prelabled and sensitized cells ($1x10^6$ cells) added with phosphatidylserine (PS, 15μg/ml) were stimulated at 37℃ for 10min by specific antigen(0.1μg/ml OA) or PS alone in a final 200$\mu\ell$ volume in 5ml polypropylene tubes. Ethanol or butanol (0.5% v/v) was added at various time before stimulation. Reactions were stopped by adding 2ml cold TGCM and centrifuged for 10min at 800 x g. Cellular lipids were extracted from the cell pellet by using a modification(18) of the Bligh and Dyer procedure(34). In experiments utilizing NY945, cells were always ncubated concomitantly with anti-OA after prelabeling, and also incubated concomitantly with 0.1μg/ml OA.

Separation of labeled phospholipids by TLC

A double one-dimensional TLC(Polygram precoated silica gel G plates, 10 x 20cm) was used to separate phospholipids of interest from extracted lipids. After they had developed, the 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 and counted in scintillation vials containing 5ml of scintillation cocktail.

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(34). 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 0.41, and that for PBut was 0.81.

Measurement of mass 1,2-diacylglycerol

Prelabeled cells (1-1.25 x 10^6) with [^{3}H]myristic acid (1μM, 1μCi) were sensitized by anti-OA (1 ml/10^6 cells) at 37°C for 45 min and stimulated by 0.1μg/ml OA at 37°C for 10 min as described(29). The labeled lipids were extracted by Bligh and Dyer method(34). The standard with extracted samples were 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. Both Rf value for DAG after prelabeling with [^{3}H]myristic acid were 0.55.

Determination of phospholipid methylation

Phospholipid methylation was determined as described in Ishizaka et al(35). The purified mast cells sensitized with anti-OA(~4x10^5 cells) were incubated(25 min, 37°C) with L-[^{3}H]methyl methionine(2μm, 165μCi/ml). The resuspended cells were challenged with 0.1μg/10μl OA for the time specified. The reaction was stopped by the addition of 900μl of ice-cooled 10% TCA containing 10mM L-methionine. The precipitates were washed with 10% TCA and then extracted with 3ml of chloroform/methanol(2:1), v/v). The chloroform phase was washed twice with 1.5ml of 0.1M KCL in 50% methanol. The chloroform phase was evaporated to dryness at room temperature. The residue 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 Aole extract was effective on the anti-inflammation 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 100μg/0.2ml of OA-Al(OH)$_3$ strongly had the symptoms of anaphylactic shock and finally died. When pretreated with more than 3 mg/kg of W1P, any symptoms of anaphylactic shock was not observed (Table 1).

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

We examined the effect of NY945 on the histamine and leukotriene releases from guinea pig lung mast cells activated with OA-anti-OA complexes. When the mast cells sensitized with anti-OA antibody were challenged by 0.1μg/ml OA after pretreatment of NY945, 10μg/ml, histamine release showed a 69.5% decrease when compared to the OA alone. The amount of leukotriene released by 10μg/ml NY945 pretreatment showed a 27.3% decrease when compared to OA alone group. The inhibitory effect of both mediator release evoked by NY945 pretreatment showed the dose-dependent response of NY945.

The effect of NY945 on the phospholipase D activity during mast cells activation.

In the mast cells sensitized only with 0.1μg/ml of OA, the PEt or PBut 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. However, the production of PEt showed significant decrease after NY945 (10μg) pretreatment. PBut produced by NY945 pretreatment deceased up to 48% when compared to antigen alone.

When tyrphostin (10^{-4}M), which is known to be able to inhibit tyrosine kinase activity, was pretreated in mast cells activated by OA-anti-OA complexes, it decreased the production of PBut

The efeect of NY945 on the production of mass 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 produced at 5-10 sec and prolonged increase of DAG for more than 30 min during mast cell activation. As NY945 strongly reduced the increasing activity of PLD caused by activating mast cells with specific antigen-antibody complexes, 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 with indirect pathway system. Therefore, we examined the effect of NY945 on the production of DAG during mast cell activation. When the mast cells ($1x10^6$ cells) sensitized with anti-OA were labeled with [^{3}H]myristic acid, and challenged with 0.1μg/ml OA for 30 sec(initial increase) or 10min(secondary increase), the amount of mass DAG were 14214 $\pm$ 958 cpm or 11218 $\pm$859 cpm, respectively. The

pretreatment of NY945 decreased up to 35.9% and 50.6% (9111 ± 389 cpm or 6704 ± 545 cpm). When the tyrphostin (10^{-4}) was pretreated in the mast cell activation, it unexpectedly inhibited the initial increase of DAG production up to 28.1% (102`4 ± 241 cpm) as well as it inhibited the continuous increase of DAG production up to 32.6% (7561 ± 469 cpm).

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

The incorporation of the[3H]methyl moiety into the phospholipids reached a maximum at 15 sec after the addition of OA, and phospholipid methylation decreased rapidly in 1 min. The inhibition of methylation with NY945(50μg) was 44% at 15 sec after challenging of OA.

CONCLUSION

From these results, it can be inferred that NY945 inhibits histamine release by inhibiting of DAG production from phospholipids during mast cell activation, which mediated via phosphatidylinositide-PLC and phosphatidylcholine-PLD systems or via the systems of phosphatidylinositide-PLD and phosphatidylcholine-PLD subtypes. It can also be inferred that PLD or PLC system is related with tyrosine kinase activity. 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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Table 1. Experimental design of active systemic anaphylaxis, and symptoms of BALb/c mice after OA-Al(OH)$_3$ challenge[a].

Group[2]	Pre-treatment	Sensitizing Antigen	Challenging Antigen	Severing of Anaphylaxis				
				-	±	+	++	+++
1	Saline	Saline	Saline	8	-	-	-	-
2	Saline	OA-A1(OH)$_3$ (100 μg/0.2 mℓ)	OA-A1(OH)$_3$ (100 μg/0.2 mℓ)	-	-	-	-	8
3	Aloe							
	1mg/kg	"	"	1	4	3	-	-
4	2mg/kg	"	"	4	3	1	-	-
5	3mg/kg	"	"	8	-	-	-	-
6	5mg/kg	"	"	8	-	-	-	-

a. Each group was injected by ip and iv following experimental design for 7wks, and observed the degree of symptoms, the same as described in "Material and Method".

b. Number of animal in each group was 8 mice.