Inhibitory Mechanism of Aloe Single Component (Alprogen) on Mediator Release in Guinea Pig Lung Mast Cells Activated with Specific Antigen-Antibody Reactions

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ABSTRACT

We previously reported that the glycoprotein extracted from aloe strongly inhibited the mediator releases caused by the activation of guinea pig lung mast cells. Therefore, this study aimed to purify a single component that has an anti-inflammatory effect from crude aloe extract and then to assess the effects of aloe single component (alprogen) on the mechanism of mediator releases caused by the mast cell activation. We purified aloe extracts by using various columns. We also purified mast cells from guinea pig lung tissues by using enzyme digestion, rough and discontinuous density Percoll gradient. Mast cells were sensitized with IgG1 (anti-ovalbumin) and challenged with ovalbumin. Histamine was assayed by using a fluorometric analyzer and leukotrienes by radioimmunoassay. [Ca^{2+}], level was analyzed by using a confocal laser scanning microscope. Protein kinase activity was determined by the protein phosphorylated with [γ-32P]ATP. The phospholipase D activity was assessed by the labeled phosphatidylalcohol. The amount of mass 1,2-diacylglycerol (DAG) was measured by the [3H]DAG produced when prelabeled with [3H]myristic acid. Phospholipase A	extsubscript{2} activity was determined by measuring the lyso-phosphatidylcholine released from the labeled phospholipids. Alprogen significantly decreased histamine and leukotriene releases and blocked completely Ca^{2+} influx during mast cell activation. The protein kinase C and phospholipase D activities were decreased by alprogen in dose-dependent manner. Alprogen inhibited mass DAG formation and the phospholipase A	extsubscript{2} activity during mast cell activation. The data suggest that alprogen purified from aloe inhibits multiple signals as well as blocking Ca^{2+} influx caused by mast cells activated with specific antigen-antibody reactions and that the inhibition of histamine and leukotriene release follows.

Plant medicines have been commonly used in basic health care in many countries throughout the centuries. One of the fairly well documented preparations in traditional medicine is the whole or parenchymous leaf gel of aloe vera (Grindlay and Reynolds, 1986). Aloe vera is a complex plant containing many biologically active substances (Klein and Penneys, 1988). It has been reported that glycoprotein extracted by aloe vera has a strong anti-inflammatory response (Davis, 1988; Davis et al., 1991, 1992; Shelton, 1991) and antiallergic reactions (Ro et al., 1998b), and that the polysaccharides, especially mannose-6-phosphate, in aloe vera have strong wound healing activity and an 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 the consequence of the release of granular mediators (histamine, 5-hydroxytryptamine, etc.), newly synthesized mediators (leukotrienes, prostaglandins, 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. The activation of tyrosine kinase (Jouvin et al., 1994; Blank et al., 1995), phospholipase C (PLC) (Berridge and Irvine, 1984), phospholipase D (PLD) (Gruchalla et al., 1994b; Gruchalla et al., 1995), and phospholipase A	extsubscript{2} (PLA	extsubscript{2}) (Berridge and Irvine, 1984) and the cross-linking of receptor-bound IgE antibody by a specific antigen. The activation of tyrosine kinase (Jouvin et al., 1994; Blank et al., 1995), phospholipase C (PLC) (Berridge and Irvine, 1984), phospholipase D (PLD) (Gruchalla et al., 1994b; Gruchalla et al., 1995), and phospholipase A	extsubscript{2} (PLA	extsubscript{2}) (Berridge and Irvine, 1984)

ABBREVIATIONS: PLD, phospholipase D; OA, ovalbumin; PBut, phosphatidylbutanol; DAG, 1,2-diacylglycerol; PKC, protein kinase C; PLA	extsubscript{2}, phospholipase A	extsubscript{2}; PC, phosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonyl phosphatidyl[14C]choline; S.A., specific activity.

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A<sub>p</sub> (PLA<sub>p</sub>) (Hirasawa et al., 1995), and Ca<sup>2+</sup> influx (Weintraub et al., 1994) follows. The activation of these enzymes, especially PLC or PLD activation, ultimately leads to the production of second messengers such as 1,2-diacylglycerol (DAG), which is known to be produced by protein kinase activation in a rat mast cell line (Lin et al., 1994), such as protein kinase C (PKC), which is known to be activated by DAG (Altrichter et al., 1995), and Ca<sup>2+</sup>, which is known to precede the release of mediators (Weintraub et al., 1994).

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

**Materials and Methods**

**Animals.** Hartley albino female guinea pigs, weighing about 200 to 250 g, were used. Animals were maintained in accordance with the National Institutes of Health Principles of Laboratory Animal Care.

**Drugs and Solutions.** The following substances were used. Ovalbumin (OA), collagenase (type I), elastase (type I, porcine pancreatic), arachidonic acid, Tris-HCl, silver nitrate, a polypeptide size marker, phosphatidylcholine (PC), lyso-PC, methyl-α-D-mannopyranoside, Fluor-3 AM, and polyvinylpyrolidone were all purchased from Sigma Chemical Co. (St. Louis, MO); gelatin was purchased from Difco Laboratories (Detroit, MI); Percoll was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden); LK5DF and LK6D silica gel plates were purchased from Whatman (Maidstone, Kent, UK); [9,10α-3H]palmitic acid [specific activity (S.A.), 53.0 Ci/mmol], [3H]myristic acid (S.A., 51 Ci/mmol), [γ-32P]ATP (S.A., 3000 Ci/mmol), leukotriene D<sub>4</sub> (LTD<sub>4</sub>) assay kit, and 1-palmitoyl-2- arachidonoyl phosphatidyl-[14C]choline (PAPC; S.A., 40–60 Ci/mmol) were all purchased from NEN (Seoul, Korea); and phosphatidylbutanol (PBut) was purchased from Avanti Polar Lipids, (Albaster, AL). Sephadex G-25, DEAE-Sephacryl, concanaavalin A Sepharose, Superdex 75, phenyl Sepharose CL-4B, and Sephacryl-100 HR was purchased from Amersham Pharmacia Biotech (Upssala, Sweden), and POROS 50 HQ was purchased from Boehringer Mannhein Co. (Mannheim, Germany). All chemicals used in these studies were of the highest grade available.

**Purification of Aloe Vera.** Fresh aloe vera leaves (10 kg) were crushed with a commercial blender (LG Electric Co., Seoul, Korea) in 1/3 volumes of extraction buffers (50 mM sodium phosphate, pH 8.0, 1.44 mM 2-mercaptoethanol, 1% polyvinylpyrolidone, and 1 mM EDTA) and the slurries were collected. The slurries were filtered through cheesecloth and centrifuged at 10,000 g for 30 min at 4°C. The supernatant of the crude extracts was precipitated with 25 to 80% ammonium sulfate saturation. The precipitate was dissolved in 20 mM Tris-HCl (pH 8.0) buffer and was then desalted by application to a Sephadex G-25 column (8.0 × 80 cm) equilibrated with the same buffer. The desalted extracts were applied to a DEAE-Sephacel column (3.4 × 30 cm) equilibrated with 20 mM Tris-HCl (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, and adjusted to 0.5 M NaCl in 20 mM Tris-HCl (pH 7.4).

The eluted proteins were chromatographed through a column of concanaavalin A-Sepharose (3.4 × 26 cm) equilibrated and washed with 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 M NaCl. The eluates (negative charged proteins in the pH 8.0 precipitate) were recovered in the pass-through fraction and concentrated by ultrafiltration and dialyzed by 20 mM Tris-HCl (pH 7.4) buffer. Bound glycoproteins were eluted with a 0.5 M methyl-α-D-mannopyranoside in the same buffer, dialed against 20 mM Tris-HCl (pH 7.4) buffer, and applied to a POROS 50 HQ anion exchange column that had been equilibrated with the same buffer. After washing with the same buffer, proteins were eluted at the flow rate of 4 ml/min with a 400-mL linear gradient of 0.0 to 0.5 NaCl in the same buffer. Fractions containing antihistaminic activity were collected and concentrated to 1.5 ml using and Amicon Centriplus 10 concentrator (Amicon, Beverly, MA). The preparation was filtered through Superdex 75 column with 20 mM Tris-HCl (pH 7.4) buffer containing 50 mM NaCl. Flow rates were 3.0 ml/min. Fractions containing antihistaminic activity were applied to a phenyl Sepharose CL-4B hydrophobic interaction chromatography column (2 ml of total volume) that had been equilibrated with 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 M (NH4)2SO4. After washing with the same buffer until only basal levels of proteins were detected, the column was eluted at the flow rate of 0.4 ml/min with 40 mL decreasing linear gradient of 0.5 to 0 M (NH4)2SO4 in the buffer. Fractions containing antihistaminic activity were collected, and it is coined as alprogen.

**Polyacrylamide Gel Electrophoresis (PAGE).** Electrophoresis of total proteins under the denaturing condition was performed by the modified procedure described by Shagger and Jagow (1987). SDS-PAGE gel included a 0.4% stacking gel, a 10.0% space gel, and a 16.5% separating gel, and the space and separated gels were stained with silver nitrate.

**Active Sensitization Protocol (Anti-OA Production).** Ten outbred female guinea pigs were first immunized by footpad injections of mixture of 50 μg OA and complete Freund’s adjuvant. One week after, 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 1 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 was determined as described in a previous article (Adersson, 1980). Serum IgG<sub>1</sub> antibody was separated by affinity column chromatography. Guinea pig blood serum was applied to anti-IgG<sub>2</sub> affinity column and 0.1M citric acid (pH 2.1) was used to wash the column. IgG<sub>1</sub> was passed through and the absorbed IgG<sub>2</sub> antibody was rinsed by 0.2 M sodium carbonate (pH 11.3). The separated IgG<sub>1</sub> was concentrated under pressure for the experiment. The titers of anti-OA were 1600 to 3200 (dilution time). 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; Ro et al., 1998a).

Brieﬂy, lungs obtained from 16 unsensitized guinea pigs were each perfused with 50 ml of the modiﬁed Tyrode’s buffer (T9CM buffer) consisting of 137 mM NaCl, 0.36 mM NaH2PO4, 2.6 mM KCl, 1 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 119 mM NaHCO<sub>3</sub>, 5.5 mM glucose, and 1 g/l gelatin, pH 7.4. After removing large airways and blood vessels, the lungs were minced with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Gomshall, England). Pooled tissue was treated three times with collagenase (125 U/g tissue) and elastase (5 U/g tissue). Time of each consecutive exposure of lung fragments to the enzymes was 15, 15, and 25 min, respectively. Freed cells were separated from residual tissue by ﬁltration through mesh and Nytex mesh (100 μm). The cells were washed with Tyrode’s buffer without CaCl<sub>2</sub> and MgCl<sub>2</sub> containing gelatin (TG buffer) and layered over gradients consisting of 10 ml of Percoll (density, 1.045/ml) and centrifuged at 800g for 20 min. Pelleted cells (containing mast cells) were resuspended in TG buffer and applied for further purification using 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 800g for 20 min (~3.5 × 10<sup>6</sup> cells/gradiant). The cell band obtained between the 1.09 and 1.10 g/ml densities contained the highest purity and number (1–2 × 10<sup>8</sup>) of mast cells. This gradient band was removed, washed with T9CM 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 80 to 90%.

**Mediator Release from Mast Cells.** The 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 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 using alprogen (0.5, 1.0, 2.5, and 5.0 μg/4 × 10^5 cells), we conducted four experiments. In the first experiment alprogen was added to mast cells sensitized with anti-OA (1 × 10^6 cells/1 ml anti-OA antibody). In the second experiment alprogen was added to mast cells 5 min before OA challenge (0.1 μg/ml). In the third experiment alprogen was added to mast cells activated with OA and anti-OA reactions. In the fourth experiment alprogen was concomitantly added to mast cells with OA antigen (Undem et al., 1985; Ro et al., 1991).

**Histamine Assay.** Histamine was analyzed by the automated fluorometric method (with a dialyzer) as described by Siraginan (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.** The leukotriene content of each cell supernatant was determined by radioimmunoassay as described previously (Aharony et al., 1983; Ro et al., 1991). The leukotriene antibody was diluted in buffered saline (5 mM 2- (N-morpholino)ethanesulfonic acid and HEPES adjusted to pH 7.4 with 1 N NaOH) containing 0.1% gelatin. Each assay tube contained 100 μl of sample supernatant, antibody (50 μl of a 1:1000 dilution), and 50 μl of [3H]LTD_4 (2500–3000 cpm) in buffered saline. Incubations were for 2 hr 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 minutes after incubation, the mixture was centrifuged at 800 g at 4°C and 0.4 ml of the supernatant was added to Aquasol (NEN Research Products) for counting by liquid scintillation spectrometry (model 3225; Hewlett-Packard, Palo Alto, CA). Standard curves were constructed in the presence of antigen using LTD_4. The detection limit of the assay was 0.045 pmol LTD_4 released as expressed as pmol/4 × 10^5 cells.

**Determination of [Ca^{2+}], Level.** Sensitized mast cells (4 × 10^5) were incubated for 30 min after adding Fluo-3 AM (5 μM) and fixed on a glass slide treated with poly-L-lysine and OA antigen (0.1 μM) flow out on a glass slide for stimulation. The purified mast cells with Fluo-3 AM also were fixed on glass slides and then a calcium ionophore (0.5, 1.0 μM) flowed on a glass slide for stimulation. The calcium ions (Ca^{2+}), of a single mast cell was analyzed with a Confocal laser scanning microscope (Leica TCS NT Confocal Microscopy, Heidelberg, Germany) (Weintraub et al., 1994). The quantity of [Ca^{2+}], was estimated with optical density (A) using the computer software program, TINA 2.0 (Raytest Co., Straubenhardt, Germany).

**Determination of PKC Activity.** The sensitized mast cells (1 × 10^6 cells) were preincubated with histone (0.2 mg/ml), phosphatidylserine (PS; 40 μg/ml), and γ-[32P]ATP (1 μM) at 30°C for 5 min. After the stimulation of mast cells, the reaction for stimulation was stopped by adding 1 ml of 10% TCA at 4°C for 30 min. The precipitated proteins were filtered through a glass fiber disk (Whatman) to eliminate unreacted [γ-32P]ATP and washed four times with 20 mM tetrasodium pyrophosphate and once with absolute ethanol. After the glass fiber disks were dried, radioactivities were measured with a liquid scintillation counter (Altrichter et al., 1995).

**Determination of PLD Activity during Mast Cell Activation.** To label mast cell phospholipids, purified mast cells were prelabeled with [3H]palmitic acid. Purified cells (1–2 × 10^7) were suspended in a final volume of 1 ml TGCM and [3H]palmitic acid (at final concentration of 3.3 μM; 200 μCi/ml) and incubated at 37°C for 1 h. Cells were washed twice and resuspended in TGCM before using in cell activation.

Prelabeled cells (0.75–1.25 × 10^6) were sensitized by anti-OA antibody (IgG1, 1 ml antibody/10^6 cells) at 37°C for 45 min, and washed and resuspended in TGCM. Prelabeled and sensitized cells (1 × 10^6 cells) added with 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 polypolyethylene tubes. Butanol (50 mM) was added 5 min before stimulation. Reactions were stopped by adding 2 ml cold TGCM and centrifuged for 10 min at 800g. 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 using alprogen, cells were preincubated with alprogen for 5 min before challenge of 0.1 μg/ml OA.

The standards (phosphatidic acid and PBut) with extracted samples for the measurement of [3H]PBut were applied to the oxalated-treated thin-layer chromatography (TLC) plates (LK6D silica gel 60; Whatman) plates (presorbed-TLC, 5 g potassium oxalate dissolved in 250 ml H_2O, made up to 500 ml with methanol), developed to the top of the TLC plates with ethyl acetate/acetate acid/2,2,4-trimethylpentane (9:2:5) and visualized with iodine staining. The RF value for PBut was 0.81. TLC plates were scraped and counted. Butanol was used because of the 5-fold lower potency of ethanol as an acceptor in the transphosphatidylation reaction.

**Determination of Mass DAG during Mast Cell Activation.** Prelabeled cells (1–2.5 × 10^6) with [3H]myristic acid (0.1 nM, 1.0 μCi) 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 10 min (Lin et al., 1994). The reaction was stopped by adding 1 ml of methanol. The labeled lipids were extracted by the Bligh and Dyer method (1959). The standard with extracted samples was applied to the presorbed-TLC plates (LK5DF) and developed up to the half of the TLC plates with ethyl acetate/acetate acid/trimethylpentane (9:2:5). After air drying the TLC plates were filled to the top in a second system with hexane/diethylether/methanol/acetate acid (90:20:3:2). The location of [3H]DAG was checked by exposure to iodine vapor. The TLC plates were scraped to measure radioactivity. Alprogen was added at 5 min before antigen challenge. The RF value for DAG was 0.55.

**Determination of PL_{A2} Activity.** Sensitized mast cells (1 × 10^6) were preincubated with [3H]PAPC (200 μCi, 1 μM) and phospholipid at 37°C for 5 min. Mast cells prelabeled were washed twice and resuspended in TGCM buffer. The cells were sensitized and stimulated, and the reaction was stopped by the addition of 10% TCA. The cell suspension was mixed with 0.2 ml of n-butanol. The lipid was extracted as described above. The lipids were spotted onto silica gel and then developed with chlororform/ethanol/water/triethylamine (30:34:8:8:35, by vol). Lyso-PC, PC, and arachidonic acid were used as standards. The amount of lyso-PC that is produced by the product of [4H]PC was measured with a liquid scintillation counter (Hiratsawa et al., 1995).

**Statistical Analysis.** Experimental data are shown as mean ± S.E. An ANOVA was used for statistical analysis. An analysis of significance between each control group and experimental group was carried out with the Scheffe’s method. p values < .05, .01, .001 were considered significant.

**Results**

**Purification of Single Component, Alprogen, from Crude Aloe Extracts.** We attempted to purify aloe vera as described in Materials and Methods. We obtained alprogen that showed as a single band by SDS-PAGE and silver stain-
ing, and we assumed it was glycoprotein. The molecular mass of alprogen was estimated to be approximately 10.0 kDa on SDS-PAGE (Fig. 1). The isoelectric point (pl) value for alprogen has been estimated to be 6.0 by using ampholine polyacrylamide gels. However, further studies on the characteristics of alprogen are needed. The N-terminal 15-amino acid sequence analysis of alprogen was NEVPYLRRTGEVLGPN. The protein was found not to be homologous by homology search in GenBank, although it is similar to vesicular γ-aminobutyric acid transporter, sulfate transporter, aminopeptidase, etc.

Effect of Alprogen on Mediator Releases during Mast Cell Activation. To examine whether alprogen 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 alprogen (0.5, 1.0, 2.5, 5.0, or 10.0 μg/ml). The mast cells sensitized with anti-OA antibody were also challenged by varying concentrations of alprogen (0.5, 1.0, 2.5, 5.0, or 10.0 μg/ml). The results showed that alprogen itself did not affect the mediator releases (data not shown).

We conducted three experiments. In the first experiment alprogen was added to mast cells sensitized with anti-OA. In the second experiment alprogen was added to mast cells challenged with OA. In the third experiment alprogen was added to mast cells activated with OA and anti-OA reactions. In all three experiments, histamine release was similar: 19.5 ± 0.66% (with anti-OA), 18.8 ± 0.87% (with OA), and 19.3 ± 0.28% (OA-anti-OA), respectively. This means that the decrease of histamine release was 32.7, 35.2, and 33.4%, respectively, compared with the OA alone which was 29.0 ± 1.0%. Leukotriene release was also affected similarly to the histamine release in all three experiments. However, when the alprogen was concomitantly added with OA challenge (fourth experiment), the inhibitory effect of mediators reduced by approximately 15%. Therefore, alprogen was added to mast cells 5 min before OA challenge.

When the mast cells sensitized with anti-OA antibody were challenged by 0.1 μg/ml OA after the pretreatment of 1.0 μg/ml alprogen, histamine release was 18.6 ± 1.90% and that showed a 35.9% decrease when compared with the OA alone, which was 29.0 ± 1.10%. The amount of leukotriene released by 1.0 μg/ml alprogen pretreatment was 29.8 ± 5.6 pmol/10^6 cells, which was a 30.5% decrease compared with the 40.5 ± 7.50 pmol/10^6 cells of OA alone group (Table 1). The inhibitory effect of both mediator releases by alprogen pretreatment showed the dose-dependent manner. In this study, the 1.0- and 5.0-μg doses of alprogen were used in each experiment, because in both mediators, release of the activated mast cells was decreased by over 35 and 50%, respectively.

When mast cells were activated with a calcium ionophore (0.5 and 1.0 μM), histamine release was 47.7 ± 1.5% and 62.6 ± 1.2%, respectively. Leukotriene released by a calcium ionophore (0.5 and 1.0 μM) was 39.6 ± 5.69 pmol/4 × 10^5 cells and 52.7 ± 8.56 pmol/1 × 10^6 cells, respectively. However, alprogen (5 μg) did not inhibit histamine and leukotriene releases evoked by a calcium ionophore (0.5 and 1.0 μM).

Effects of Alprogen on Influx of Ca^2+ during Mast Cell Activation. It has been reported that the increase of [Ca^2+] i, and the activation of PKC are necessary for degranulation of preformed inflammatory mediators in mast cells (Takata et al., 1994). Therefore, the effects of alprogen on [Ca^2+] i were examined in a single mast cell and visualized by using a confocal laser scanning microscope through fluorescence intensity (optical density, A). [Ca^2+] i was significantly decreased by alprogen (0.5 μg/ml) with OA (Table 1).

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TABLE 1

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<thead>
<tr>
<th>Treatment</th>
<th>Histamine</th>
<th>Leukotrienes</th>
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<tr>
<td></td>
<td>%</td>
<td>pmol/10^6 cells</td>
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<tr>
<td>OA alone</td>
<td>29.0 ± 1.10</td>
<td>42.9 ± 5.40</td>
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<tr>
<td>Alprogen (μg)</td>
<td>23.5 ± 1.21*</td>
<td>40.5 ± 7.53</td>
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<tr>
<td>0.5</td>
<td>18.6 ± 1.90***</td>
<td>29.8 ± 5.62***</td>
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<tr>
<td>2.5</td>
<td>11.1 ± 6.12***</td>
<td>25.2 ± 7.85***</td>
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<tr>
<td>5.0</td>
<td>7.5 ± 7.50***</td>
<td>25.2 ± 6.23***</td>
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*p < .05; **p < .01; ***p < .001 compared with OA alone.
Effect of Alprogen on PKC Activity during Mast Cell Activation. Because $[\text{Ca}^{2+}]_i$ is decreased by the treatment of alprogen, alprogen may also influence PKC activity. Phosphorylated protein was monitored to measure the effects of alprogen on the activity of PKC. Phosphorylated proteins activated by OA and anti-OA reactions increased by approximately 7.8 times (from 27,793 ± 10,200 to 217,755 ± 20,334 cpm). In addition, 1 mg of alprogen decreased the activity of PKC by 37.4% (from 217,755 ± 20,334 to 136,232 ± 9,432 cpm) and 5.0 mg of alprogen decreased it by 51.3% (from 217,755 ± 20,334 to 106,118 ± 19,428 cpm; Fig. 3).

Effect of Alprogen on Activation of PLD during Mast Cells Activation. An increase of membranous PLD activity during mast cell activation evoked by specific antigen-antibody reactions ultimately leads to the release of mediators from mast cells. Therefore, the effect of alprogen on the formation of mass DAG in the lung mast cells activated by OA and anti-OA reactions was investigated. Mass DAG production decreased by 40% after the treatment with alprogen.
(1.0 μg) (from 36,200 ± 900 to 21,900 ± 30 cpm; Fig. 5). In the treatment of alprogen (5.0 μg), the formation of mass DAG is completely blocked.

Effect of Alprogen on Activation of PLA2 during Mast Cell Activation. The increase of [Ca\(^{2+}\)]i has a number of effects on mast cells. One of the major effects is the induction of the association of PLA2 with membranes to facilitate the synthesis of lipid mediators such as leukotrienes and prostaglandins. Because the release of arachidonic acid is responsible, in part, for type I hypersensitivity reactions, we investigated whether alprogen inhibits PLA2 activity. As shown in Fig. 6, the activity of PLA2 increased by about 41% in mast cells activated by 0.1 μM OA challenge (from 8,237 ± 554 to 11,617 ± 769 cpm). However, the activity of PLA2 decreased by 26.7% (from 11,617 ± 769 to 8,520 ± 1,144) by the treatment with 5.0 μg/ml alprogen compared with that of OA challenge alone.

Discussion

It has been reported that aloe vera has a stimulatory system that enhances antibody production (Shelton, 1991), and an inhibitory system that influences both inflammatory and immune responses (Davis et al., 1991, 1992, 1994a). The interaction between these two systems is referred to as biological modulation. In this study we focused on the inhibitory system of aloe vera.

A single inhibitory component in aloe vera was purified by various processes. We obtained alprogen that showed a single band by using SDS-PAGE (Fig. 1), and we assumed it to be glycoprotein.

In this experiment, we observed that alprogen strongly inhibits histamine and leukotriene releases during the activation of mast cells by specific antigen-antibody reactions (Table 1). The inhibitory effects of mediator releases by alprogen has a kind of anti-inflammatory activity found in the extracts of aloe vera (Davis et al., 1991, 1992, 1994a,b).

To examine the binding sites of alprogen, we conducted three different experiments as described in Effect of Alprogen on Mediator Releases during Mast Cell Activation in Results. We found similar results in all three experiments except one (fourth experiment). From these results, it can be inferred that alprogen binds to the mast cell surface differently from the antibody binding site, and that it then blocks the movement of antibody-bound receptor to cross-link to antigen. It can also be inferred that alprogen binds to a variable region of IgG\(_i\) antibody and then blocks the binding of antigen to the variable region.

We first attempted to confirm the inhibitory mechanism of alprogen on the mediator release caused by mast cells activated with specific antigen-antibody reactions. When mast cell membrane receptors are activated by antigen-antibody reactions, Ca\(^{2+}\) long has been recognized as essential for secretion from mast cells. [Ca\(^{2+}\)]i increased remarkably in the presence of external Ca\(^{2+}\) after stimulation with antigen-antibody reactions (Fig. 2a). This is the same result reported previously in a rat mast cell line (Hide and Beaven, 1991). The increase in [Ca\(^{2+}\)]i due to influx from extracellular Ca\(^{2+}\) by OA and anti-OA reactions was blocked by alprogen (Fig. 2, c–d), but the increase in [Ca\(^{2+}\)]i by a calcium ionophore was not blocked. Although Ca\(^{2+}\) alone is not sufficient to trigger exocytosis, an increase in [Ca\(^{2+}\)]i is prominent signal in mast cells. Therefore, it can be inferred that alprogen inhibits, in part, the release of mediators by blocking Ca\(^{2+}\) influx caused by specific antigen-antibody reactions but does not block the mediators by nonimmunological reactions (with a calcium ionophore). Also, alprogen moves out Ca\(^{2+}\) more rapidly than in the absence of external Ca\(^{2+}\). This result needs more study.

There are a number of reports that PKC is an essential transducer of signals for secretion in human and RBL-2H3 mast cells (Warner and MacGlashan, 1990; Ozawa et al.,

![Fig. 5. Effects of alprogen on the mass DAG formation during the activation of mast cells sensitized with anti-OA. Purified mast cells (1 × 10\(^6\)) were labeled with \(^{[3H]}\)myristic acid (0.1 nM, 1.0 μCi) for 1 h, sensitized with anti-OA antibody (1 ml/10\(^6\) cells) for 45 min, and then challenged with OA (0.1 μg/ml) for 10 min in the absence or presence of alprogen (1.0 and 5.0 μg). \(^{[3H]}\)DAG was extracted and separated from other lipids as described in Materials and Methods. **P < .01, ***P < .001 compared with OA alone. ††P < .01, †††P < .001 compared with OA alone.

![Fig. 6. Effect of alprogen on PLA2 activity in the activation of purified guinea pig lung mast cells sensitized with anti-OA antibody. Purified and sensitized mast cells (1 × 10\(^6\) cells) were prelabeled with \(^{[14C]}\)PAPC (200 μCi, 1.0 μM) sensitized with anti-OA and then challenged with OA (0.1 μg/ml). The labeled phospholipids were determined by the methods described above. *P < .05 by comparison without OA challenge; †P < .05 compared with OA challenge.]
We previously reported that protein is phosphorylated by PKC in the guinea pig lung mast cell activation (Ro et al., 1998b). Some PKC isozymes are activated by the increase in 

\[ \text{[Ca}^{2+}\text{]} \], and by DAG, which is the result from cell activation. Therefore, the decrease in 

\[ \text{[Ca}^{2+}\text{]} \], caused by alprogen may influence the PKC activity. Alprogen remarkably inhibited PKC activity (Fig. 3). As a result, it can be inferred that alprogen blocks external Ca\textsuperscript{2+} influx, followed by the inhibition of PKC activity.

When mast cell membrane receptors are activated by antigen-antibody reactions, Ca\textsuperscript{2+}-dependent enzyme systems in the cell membrane are activated. The activated enzymes (PLD, PLA\textsubscript{2}, etc.) are intimately related to the generation of inflammatory mediators and synthesis of newly formed mediators, which then can induce asthma and allergic hypersensitivity. The most common second messenger that is related to histamine release is DAG. DAG can be formed either from PC indirectly by a PLD-initiated pathway or directly from other phospholipids by the activation of PLC with receptor-mediated cell activation (Gruchalla et al., 1990; Lin et al., 1992). Recently, it has been reported that the amount of DAG produced by PLD during the activation of rat peritoneal mast cells was greater than that by PLC activity (Gruchalla et al., 1990; Andrew et al., 1996). Therefore, we focused on PLD activity to examine the inhibitory mechanism of alprogen on the histamine release. It has previously been reported that PLD activity increased two to three times during guinea pig lung mast cell activation (Lin et al., 1992; Ro et al., 1998a). This increased PLD activity is decreased by alprogen pretreatment (Fig. 4). From these results it can be inferred that alprogen inhibits PLD activity during the activation of mast cells sensitized with specific antigen-antibody reactions. The inhibition of PLD activity was stronger than the inhibition of histamine release. Lin et al. (1992) suggested that the activation of PLD may be regulated by both the activation of PKC and a rise in \[ \text{[Ca}^{2+}\text{]} \]. Therefore, it can be inferred that both the decrease of PKC and \[ \text{[Ca}^{2+}\text{]} \], caused by alprogen inhibits more PLD activity. However, this result needs more study.

Because the inhibition of PLD activity by alprogen was observed, the effects of alprogen on the mass DAG production was examined. Mass DAG produced by the activation of phospholipases are completely blocked by alprogen (Fig. 5). From these results it can be inferred that alprogen inhibits DAG production via phospholipase pathways. As a result, histamine release is reduced.

The increase of \[ \text{[Ca}^{2+}\text{]} \] has a number of effects on mast cells. One of the major effects is the induction of the association of PL\textsubscript{A\textsubscript{2}} with membranes to facilitate the synthesis of lipid mediators such as leukotrienes and prostaglandins. That is, synthesis of leukotrienes is mediated by PL\textsubscript{A\textsubscript{2}} and Ca\textsuperscript{2+} mobilization, but they are not dependent on the activity of PKC (Hirasawa et al., 1995). Alprogen inhibited the activity of PL\textsubscript{A\textsubscript{2}} mediating the synthesis of arachidonic acid. The release of arachidonic acid is responsible, in part, for type I hypersensitivity. It can be suggested that because alprogen reduces histamine release and synthesis and secretion of leukotrienes simultaneously, it has potential for as an anti-allergic agent because it blocks multiple signals as well as Ca\textsuperscript{2+} influx in mast cells. Our data suggest that alprogen purified from aloe inhibits multiple signals as well as blocking of Ca\textsuperscript{2+} influx caused by mast cells activated with specific antigen-antibody reactions, and the inhibition of histamine and leukotriene releases follows.

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