

ORIGINAL ARTICLE

Alginate acid has anti-anaphylactic effects and inhibits inflammatory cytokine expression via suppression of nuclear factor- κ B activation

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Clinical and Experimental Allergy

Summary

Background Alginate acid is comprised of complex polymerized polysaccharides, and can be chemically extracted from seaweed. Alginate acid has an inhibitory effect on histamine release, but its molecular mechanisms are not well understood.

Objective To investigate the effect of alginate acid on the mast cell-mediated anaphylactic and inflammatory reaction using *in vivo* and *in vitro* models and elucidate its molecular mechanisms.

Materials and method The effect of alginate acid on an allergy model was analysed by anaphylaxis, a histidine decarboxylase (HDC) assay, and a histamine assay. Cytokine production was analysed by means of ELISA. Cytokine expression was analysed by means of RT-PCR, and Western blotting. Transcription factor activity was analysed by a luciferase assay and a transcription factor-enzyme linked immunoassay.

Results Alginate acid dose dependently inhibited compound 48/80-induced systemic anaphylaxis with doses of 0.25–1 g/kg 1 h ($P < 0.01$, $n = 6$) and significantly inhibited passive cutaneous anaphylaxis by 54.8%. Alginate acid (0.01–1 μ g/mL) inhibited histamine release from serum and peritoneal mast cells ($P < 0.01$). All these effects were stronger than those of disodium cromoglycate (DSCG), the reference drug tested. Alginate acid also inhibited HDC expression and activity on the phorbol myristate acetate (PMA)+A23187-stimulated human mast cell line, HMC-1 cells. Moreover, alginate acid significantly inhibited the production of PMA+A23187-induced inflammatory cytokines, IL-1 β and TNF- α , but not that of IL-6 or IL-8. In activated HMC-1 cells, the expression level of nuclear factor (NF)- κ B/Rel A protein increased in the nucleus, whereas the level of NF- κ B/Rel A in the nucleus was decreased by alginate acid treatment. In addition, alginate acid (0.01 μ g/mL) decreased the PMA+A23187-induced luciferase activity and DNA-binding activity.

Conclusion The present results indicate that alginate acid has potent anti-anaphylactic and anti-inflammatory properties.

Keywords alginate acid, allergy, cytokines, histamine, mast cells, NF- κ B

Submitted 21 October 2005; revised 24 February 2006; accepted 27 March 2006

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Introduction

Alginate acid is a naturally occurring hydrophilic colloidal polysaccharide obtained from the various species of brown seaweed (*Phaeophyceae*). It is a linear copolymer consisting mainly of residues of 1,4-linked D-mannuronic acid and 1,4-linked L-glucuronic acid. These monomers are often arranged in homopolymeric blocks separated by regions approximating an alternating sequence of the two acid monomers [1].

Mast cells are widely distributed in the connective tissues of mammals and other vertebrates, where they are frequently located in close proximity to blood vessels [2]. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several pro-inflammatory and chemotactic cytokines such as TNF- α , IL-1, IL-4, IL-6, IL-8, IL-13, and transforming growth factor (TGF)- β 1 [3–5].

Histamine is a chemical mediator that plays a variety of biologic roles, including anaphylactic responses, inflammation, neurotransmission, and gastric acid secretion [6]. The formation of histamine from its precursor, L-histidine, is catalysed in a single step by the enzyme histidine decarboxylase (HDC) [7]. HDC is expressed in a variety of adult cell types, including mast cells/basophils, skin, platelets, histaminergic neurons in the brain, and enterochromaffin-like cells of the gastric corpus [6].

Expression of inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF- α , is dependent on the activation of a transcription factor, nuclear factor (NF)- κ B. At least five different molecules, *NFKB1* (p105/50), *NFKB2* (p100/p52), *Rel A* (p65), *Rel B*, and *c-Rel*, belong to the NF- κ B family. Most commonly, NF- κ B dimers are composed of the *Rel A* (p65) and *NFKB1* (p50) or *NFKB2* (p52) subunits [8, 9]. NF- κ B bound to a specific consensus DNA element present on the promoter of target genes initiates the transcription of various genes [10–12]. NF- κ B normally resides in the cytoplasm, where it is retained by association with I κ B protein (α , β , γ), an endogenous inhibitor [9]. However, when activated, it translocates to the nucleus, binds the DNA, and activates genes. The activation involves the phosphorylation, ubiquitination, and degradation of I κ B, leading to the nuclear migration of NF- κ B [13–15].

In the present study, we investigated the effect of alginic acid on mast cell-mediated anaphylactic and inflammatory reaction. We found that alginic acid inhibited histamine synthesis and NF- κ B activation.

Materials and methods

Materials

Fetal bovine serum, Iscove's modified Dulbecco's medium (IMDM), ampicillin, and streptomycin were purchased from GIBCO BRL, Grand Island, NY, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol myristate acetate (PMA), A23187, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) tablets substrate (ABTS), disodium cromoglycate (DSCG), and sodium alginic acid (source, *Macrocystis pyrifera*; viscosity, ~250 cP), which is composed of approximately 61% mannuronic and 39% guluronic acid, were purchased from Sigma (St Louis, MO, USA). Human recombinant (r) IL-1 β , IL-6, IL-8, and TNF- α , purified anti-human IL-1 β , IL-6, IL-8, and TNF- α , and biotin-conjugated anti-human IL-1 β , IL-6, IL-8, and TNF- α antibodies were purchased from R&D System Inc. (Minneapolis, MN, USA). NF- κ B antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HDC antibody was purchased from Research Diagnostics Inc. (Flanders, NJ, USA). RT-PCR primers were kindly donated by Bank for Cytokine Research (BCR, South Korea).

Animals

The original stock of male Wistar rats weighing 200–300 g was purchased from Dae-Han Experimental Animal Center (Daejeon, Chungnam, South Korea). The animals were housed five to 10 per cage in a laminar air flow room maintained throughout the study at 22 ± 1 °C with a relative humidity of $55 \pm 10\%$. All protocols were approved by the Institutional Animal Care and Use Committee of College of Oriental Medicine, Kyung Hee University.

Compound 48/80-induced systemic anaphylactic shock

Mice were given an i.p. injection of 8 mg/kg of the mast cell degranulator compound 48/80. Alginic (0.25–1 g/kg) acid and DSCG (0.25–1 g/kg) were dissolved in saline and administered orally 1 h before the injection of compound 48/80. Mortality was monitored for 1 h after induction of anaphylactic shock. The blood was centrifuged at 400 g for 10 min and the serum was subsequently withdrawn. Saline was used as a control for the experiments. Saline did not yield any observable effect in any of the experiments.

Passive cutaneous anaphylaxis

An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal (ID) injection of anti-DNP IgE followed 48 h later by an injection of DNP-human serum albumin (HSA) into the rat's tail vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The rats were injected intradermally with 100 μ g of anti-DNP IgE (a mean value) into each of four dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each rat received an injection of 1 mg of DNP-HSA in PBS containing 4% Evans blue (1 : 4) via the tail vein. Alginic acid (1 g/kg) and DSCG (1 g/kg) were orally administered 1 h before the challenge. Thirty minutes after the challenge, the rats were killed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 1 mL of 0.1 N KOH and 9 mL of a mixture of acetone and phosphoric acid (5 : 13). The absorbent intensity of the extraction was measured at 620 nm in a spectrometer and the amount of dye was calculated with the Evans blue measuring-line.

Preparation of rat peritoneal mast cells

Rats were anaesthetized by ether and injected with 20 mL of Tyrode buffer B (137 mM NaCl, 5.3 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄) containing 0.1% gelatin (Sigma) into the peritoneal cavity, and the abdomen was gently massaged for about 90 s. The peritoneal

cavity was carefully opened and the fluid containing peritoneal cells was aspirated using a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150 *g* for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e. macrophages and small lymphocytes. Briefly, peritoneal cells suspended in 1 mL of Tyrode buffer B were layered on 2 mL of 22.5% w/v metrizamide (density, 1.120 g/mL, Sigma) and centrifuged at room temperature for 15 min at 400 *g*. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 mL of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose) containing 0.1% bovine serum albumin (Sigma). Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Inhibition of histamine release

Purified mast cells were resuspended in Tyrode buffer A containing 0.1% bovine serum albumin for the treatment of stimulators. Mast cell suspensions (2×10^5 cells/mL) were pre-incubated for 10 min at 37 °C before the addition of compound 48/80 (5 µg/mL). The cells were pre-incubated with alginate acid (0.01–1 µg/mL) and DSCG for 1 h, and then incubated (20 min) with compound 48/80. The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400 *g* for 5 min at 4 °C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400 *g* for 5 min at 4 °C. The histamine content was measured by the OPA spectrofluorometric procedure. The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation: % Inhibition = $(a - b) \times 100/a$ where *a* is histamine release without alginate and *b* is histamine release with alginate acid.

Western blot analysis

NF-κB was analysed by Western blot analysis. Cell extracts were prepared by a detergent lysis procedure. Cells (5×10^6 cells) were scraped, washed once with PBS, and resuspended in lysis buffer (1% Triton X-100, 1% deoxycholate in PBS). Samples were vortexed for lysis for a few seconds every 15 min at 4 °C for 1 h and centrifuged at 15 000 *g* for 5 min at 4 °C. Supernatants were assayed. Samples were heated at 95 °C for 5 min, and briefly cooled on ice. Following centrifugation at 15 000 *g* for 5 min, 50 µL aliquots were resolved by a 12% sodium

dodecyl sulphate-polyacrylamide gel electrophoresis. The resolved proteins were electrotransferred overnight onto nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycine, and 20% methanol at 25 V. Blots were blocked for at least 2 h with 1 × Tris-buffered saline containing Tween 20 (TBST) containing 5% nonfat dry milk. Protein levels were analysed according to the manufacturer's instructions.

Histidine decarboxylase assay

HMC-1 cells were pretreated with or without 0.01 µg/mL concentrations of alginate acid for 1 h before stimulation. After stimulation with PMA+A23187 for 4 h, HMC-1 cells were suspended in a solution (HDC reaction buffer) of 0.1 M potassium phosphate buffer (pH 6.8) containing 0.2 mM dithiothreitol (DTT), 0.01 mM pyridoxal-5% phosphate, 1% polyethylene glycol (*M_r* = 300), and 100 µg/mL phenylmethylsulphonyl fluoride (PMSF). They were then sonicated for 20 s and centrifuged at 10 000 *g* for 15 min at 4 °C. Supernatant containing HDC protein was dialysed overnight against the HDC reaction buffer. After a brief centrifugation, an aliquot of the supernatant was incubated with 1 mL of HDC reaction buffer containing 0.25 mM L-histidine for 2 h at 37 °C. The reaction was stopped by adding 40 µL of 6.2 M perchloric acid. After a brief centrifugation, the histamine content of the supernatant was measured as described above. HDC activity was expressed as the amount of histamine formed per minute per milligram of protein. Protein was measured with bicinchoninic acid.

Assay of interleukins-1β, 6, 8, and tumour necrosis factor-α secretion

The cells were pre-treated with alginate acid (0.01–1 µg/mL) for 1 h before PMA+A23187 stimulation for 8 h. Culture supernatants were assayed for IL-1β, IL-6, IL-8, and TNF-α protein levels using the ELISA method. The ELISA was devised by coating 96-well plates of human monoclonal antibody with specificity for IL-1β, IL-6, IL-8 and TNF-α. Before subsequent steps in the assay, the coated plates were washed with PBS containing 0.05% Tween-20 (PBST). All reagents used in this assay were incubated for 2 h at 37 °C. Recombinant IL-1β, IL-6, IL-8, and TNF-α were diluted and used as a standard. Serial dilutions starting from 5 ng/mL were used to establish the standard curve. Assay plates were exposed sequentially to biotinylated human IL-1β, IL-6, IL-8, and TNF-α, and avidine peroxidase, and ABTS substrate solution containing 30% H₂O₂. The plates were read at 405 nm. The IC₅₀ was estimated by extrapolating the given data in Fig. 4

Reverse transcriptase-polymerase chain reaction analysis

The cells were pre-treated with alginate acid (0.01 µg/mL) for 1 h before PMA+A23187 stimulation for 4 h. We

performed the RT-PCR analysis for IL-1 β and TNF- α . Total RNA was isolated from HMC-1 cells according to the manufacturer's specification using an easy-BLUETM RNA extraction kit (iNtRON Biotech, Sungnam, Kyungki-do, Korea). The concentration of total RNA in the final eluate was determined by spectrophotometry. Total RNA (2.5 μ g) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using a cDNA synthesis kit (Amershampharmacia, Amersham Bioscience, Piscataway, NJ, USA). PCR was performed with the following primers for IL-1 β (5'-CCG GAT CCA TGG CAC CTG TAC GAT CA-3'; 5'-GGG GTA CCT TAG GAA GAC ACA AAT TG-3') and TNF- α (5'-CGG GAC GTG GAG CTG GCC GAG GAG-3'; 5'-CAC CAG CTG GTT ATC TCT CAG CTC-3'). GAPDH (5'-CAA AAG GGT CAT CAT CTC TG-3'; 5'-CCT GCT TCA CCA CCT TCT TG-3') was used to verify whether equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 50 °C for IL-1 β and 60 °C for TNF- α , and GAPDH, respectively. Amplified fragment sizes for IL-1 β , TNF- α , and GAPDH were 545, 355, and 446 bp, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The mRNA levels were quantitated by densitometry.

Nuclear protein extraction

After cell activation for 2 h, 5×10^6 cells were washed in 1 mL of ice-cold PBS, centrifuged at 1000 *g* for 5 min, resuspended in 400 μ L of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15 000 *g* for 30 s. Pelleted nuclei were gently resuspended in 50 μ L of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15 000 $\times$ *g* for 5 min at 4 °C. Aliquots of the supernatant containing nuclear proteins were frozen in liquid nitrogen and stored at -70 °C. Protein was determined using bicinchoninic acid (Sigma).

Transient transfection and luciferase assay

HMC-1 cells were transfected using the Profection Mammalian Transfection System-Calcium phosphate reagent (Promega, Madison, WI, USA). After 48 h, transfected HMC-1 cells (3×10^5) were plated and stimulated with PMA+A23187. Alginic acid (0.01 μ g/mL) was added 1 h before stimulation. Cells were harvested 24 h after stimulation and washed in cold PBS before lysis in 100 μ L lysis buffer (Luciferase assay kit; Promega). After vortex mixing and centrifugation at 12 000 *g* for 1 min at 4 °C, the supernatant was stored at 70 °C until use in the luciferase

assay. Twenty millilitres of cell extract and 100 μ L of the luciferase assay reagent were mixed at room temperature. Luciferase activity was measured with a MicroLumat Plus luminometer, according to the manufacturer's protocol. All transfection experiments were performed in at least three different experiments, with similar results. Total protein was assayed using bicinchoninic acid.

Transcription factor enzyme-linked immunoassay

The cells were pretreated with alginic acid (0.01 μ g/mL) for 1 h before PMA+A23187 stimulation for 2 h. We performed the transcription factor enzyme-linked immunoassay (TF-EIA) for NF- κ B. Avidine peroxidase was coated on a 96-well ELISA plate. The coated plate was washed with PBST and then blocked with 3% skim milk solution. It was subsequently incubated with 1 μ g/mL of 5'-biotinylated 21 single-strand DNA oligonucleotide sequence for 1 h at room temperature. This sequence contains the previously described NF- κ B-binding motif. The sequence used here was: 5'-AGT TGA GGG GAC TTT CCC AGG-3'. DNA-binding reaction was carried out in a total volume of 100 μ L containing 10 μ g nuclear protein extract in a buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol, 1 mM EDTA, and 1 mM DTT, for 1 h at room temperature and then washed. NF- κ B antibodies were then added at a 1 : 500 concentration in PBS containing 3% BSA for 1 h, followed by the addition of the corresponding alkaline phosphatase (AP)-coupled secondary antibody. Between each addition, the wells were extensively washed in PBST. AP activity was then detected by the addition of p-nitrophenyl phosphate (PNPP) solution (Sigma). After a 10 min incubation period, the reaction was arrested by the addition of 0.5 M H₂SO₄. Colour intensity was detected at 405 nm using an ELISA reader. AP activity was normalized to control values (unstimulated cells).

Statistical analysis of data

The experiments shown are a summary of the data from at least three experiments and are presented as the mean $\pm$ SEM. Statistical evaluation of the results was performed by a Student's independent *t*-test and ANOVA with a Tukey's *post hoc* test. The results were considered significant at a value of *P* < 0.05.

Results

Effect of alginic acid on compound 48/80-induced systemic anaphylaxis

In order to assess the contribution of alginic acid in anaphylactic reactions, we first used an *in vivo* model of systemic anaphylaxis. We used compound 48/80 (8 mg/kg) as a systemic fatal anaphylaxis inducer. After the

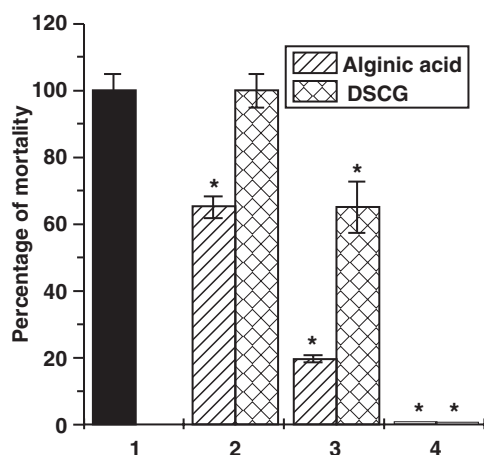


Fig. 1. Effect of alginic acid on compound 48/80-induced systemic anaphylactic reaction. Groups of mice were orally pre-treated with 200 μ L saline or alginic acid (0.25, 0.5, and 1 g/kg) 1 h before ($n = 6$ group) the compound 48/80 injection. The compound 48/80 solutions were intraperitoneally given to the group of mice. Mortality (%) within 1 h following the compound 48/80 injections is presented as the number of dead mice $\times$ 100/total number of experimental mice. 1, saline; 2, compound 48/80 + 0.25 g/kg; 3, compound 48/80 + 0.5 g/kg; 4, compound 48/80 + 1 g/kg. * $P < 0.01$; significantly different from the compound 48/80.

injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. As shown in Fig. 1, oral administration of 200 μ L saline as a control induced fatal shock in 100% of each group. When the alginic acid and DSCG were pre-treated at a concentration ranging from 0.25 to 1 g/kg for 1 h, the mortality with compound 48/80 was dose-dependently reduced. The inhibitory effect of alginic acid was higher than that of DSCG.

Effect of alginic acid on passive cutaneous anaphylaxis

Another means to test anaphylactic reactions is to induce passive cutaneous anaphylaxis (PCA). As described in the experimental procedures, local extravasation is induced by skin injection of anti-DNP IgE, followed by an intravenous antigenic challenge. Anti-DNP IgE was injected into the right dorsal skin sites. As a control, the left dorsal skin sites of the same rats were injected with saline alone. After 48 h, all rats were injected intravenously with DNP-HSA mixed with Evans blue dye. The cutaneous anaphylactic reaction was best visualized by extravasation of the dye. The inhibitory effect of alginic acid was 1 g/kg, as shown in Fig. 2.

Effect of alginic acid on histamine release and histidine decarboxylase activity from stimulated HMC-1 cells

In order to determine whether alginic acid can modulate compound 48/80-induced histamine release, mice were

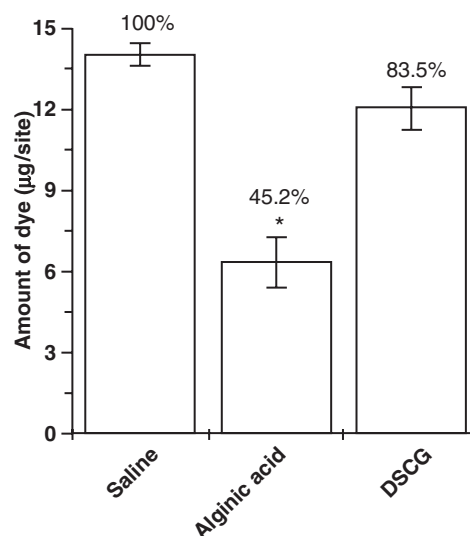


Fig. 2. Effect of alginic acid on the 48 h passive cutaneous anaphylaxis (PCA) in rats. Alginic acid (1 g/kg) and disodium cromoglycate (DSCG) (1 g/kg) were administered orally 1 h before the challenge with antigen. Each amount of dye is presented as the mean $\pm$ SEM of four independent experiments. * $P < 0.05$; significantly different from the saline value.

orally administrated with alginic acid and then intraperitoneally injected with compound 48/80. Meanwhile, rat peritoneal mast cells (RPMCs) were pre-treated with or without various concentrations of alginic acid for 1 h before stimulation. We measured the histamine using fluorescence spectroscopy at 10 min after stimulation. The inhibitory effects of alginic acid on compound 48/80-induced histamine release from serum and RPMCs are shown in Fig. 3a and b. Alginic acid inhibited compound 48/80-induced histamine release. DSCG, known as an anti-histamine drug, was used as a reference drug. The maximum inhibition rate of histamine release by alginic acid and DSCG was 60.8% and 54.9%, respectively, at 0.01 μ g/mL. We also investigated the effect of alginic acid on PMA + A23187-induced expression and activity of HDC. Alginic acid alone did not affect the unstimulated cell HDC activity. We found that alginic acid inhibited the expression and activity of HDC on PMA + A23187-stimulated cells (Fig. 3c and d). Alginic acid significantly inhibited HDC activity ($P < 0.01$). PMA + A23187 or alginic acid did not significantly affect the HMC-1 cell toxicity (data not shown).

Effect of alginic acid on phorbol myristate acetate + A23187-induced inflammatory cytokine secretion in HMC-1 cells

In order to determine whether alginic acid can modulate PMA + A23187-induced IL-1 β , IL-6, IL-8, and TNF- α secretion, the cells were pre-treated with various concentrations of alginic acid for 1 h before PMA + A23187

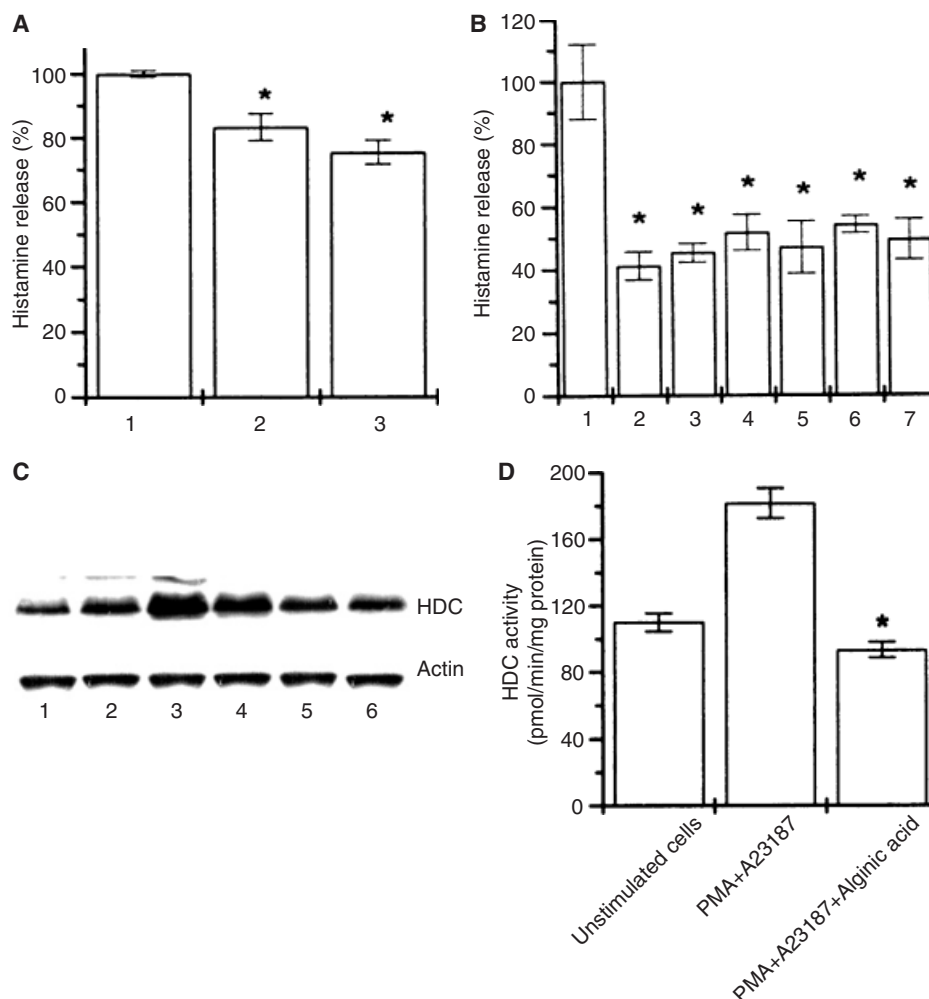


Fig. 3. Effect of alginic acid on histamine release and histidine decarboxylase (HDC) activity. Groups of mice were orally pre-treated with 200 µL saline or alginic acid (0.5 and 1 g/kg) 1 h before ($n = 6$ group) the compound 48/80 injection. Serum was isolated from blood and then assayed about histamine (a). 1, compound-48/80; 2, compound-48/80+alginic acid 0.5 g/kg; 3, compound-48/80+alginic acid 1 g/kg. Rat peritoneal mast cells were pre-incubated with drug at 37 °C for 1 h before incubation with compound-48/80. Histamine release was assayed (b). 1, compound-48/80; 2, compound-48/80+alginic acid (0.01 µg/mL); 3, compound-48/80+alginic acid (0.1 µg/mL); 4, compound-48/80+alginic acid (1 µg/mL); 5, compound-48/80+disodium cromoglycate (DSCG) (0.01 µg/mL); 6, compound-48/80+DSCG (0.1 µg/mL); 7, compound-48/80+DSCG (1 µg/mL). HMC-1 cells were pre-treated with or without 0.01 µg/mL concentrations of alginic acid for 1 h before phorbol myristate acetate (PMA)+A23187 stimulation. HDC expression and activity were evaluated by using Western blot analysis and HDC assay (c and d). Each datum is presented as the mean $\pm$ SEM three independent experiments. 1–2, unstimulated cells; 3–4, PMA+A23187; 5–6, PMA+A23187+alginic acid. * $P < 0.01$; significantly different from the control value.

stimulation. The alginic acid did not affect IL-1 β , IL-6, IL-8, or TNF- α secretion in the absence of PMA+A23187. However, in PMA+A23187-stimulated cells, IL-1 β and TNF- α secretion was significantly decreased by treatment of alginic acid ($P < 0.05$). Alginic acid inhibited PMA+A23187-induced IL-1 β secretion with IC₅₀ of 0.001 µg/mL. Alginic acid and DSCG did not affect the IL-6 or IL-8 secretion. The maximum effective concentration of alginic acid was 1 µg/mL for IL-1 β and 0.01 µg/mL for TNF- α (Fig. 4a). Cell cytotoxicity by alginic acid was negligible (data not shown). The inhibition rate of IL-1 β and TNF- α secretion was observed to be higher than that of the other inflammatory cytokines (IL-6 and IL-8) secretion. We

performed an RT-PCR analysis for IL-1 β and TNF- α at 4 h. IL-1 β and TNF- α mRNA expression was up-regulated by PMA+A23187 at 4 h, but the up-regulated IL-1 β and TNF- α mRNA expression was decreased by treatment of alginic acid (0.01 µg/mL, Fig. 4b).

The Effect of alginic acid on PMA+A23187-induced NF- κ B activation

Expression of inflammatory cytokines was regulated by a transcription factor, NF- κ B/Rel A. The expression level of NF- κ B/Rel A in the nucleus of HMC-1 cells was examined by Western blot analysis. In PMA+A23187-stimulated

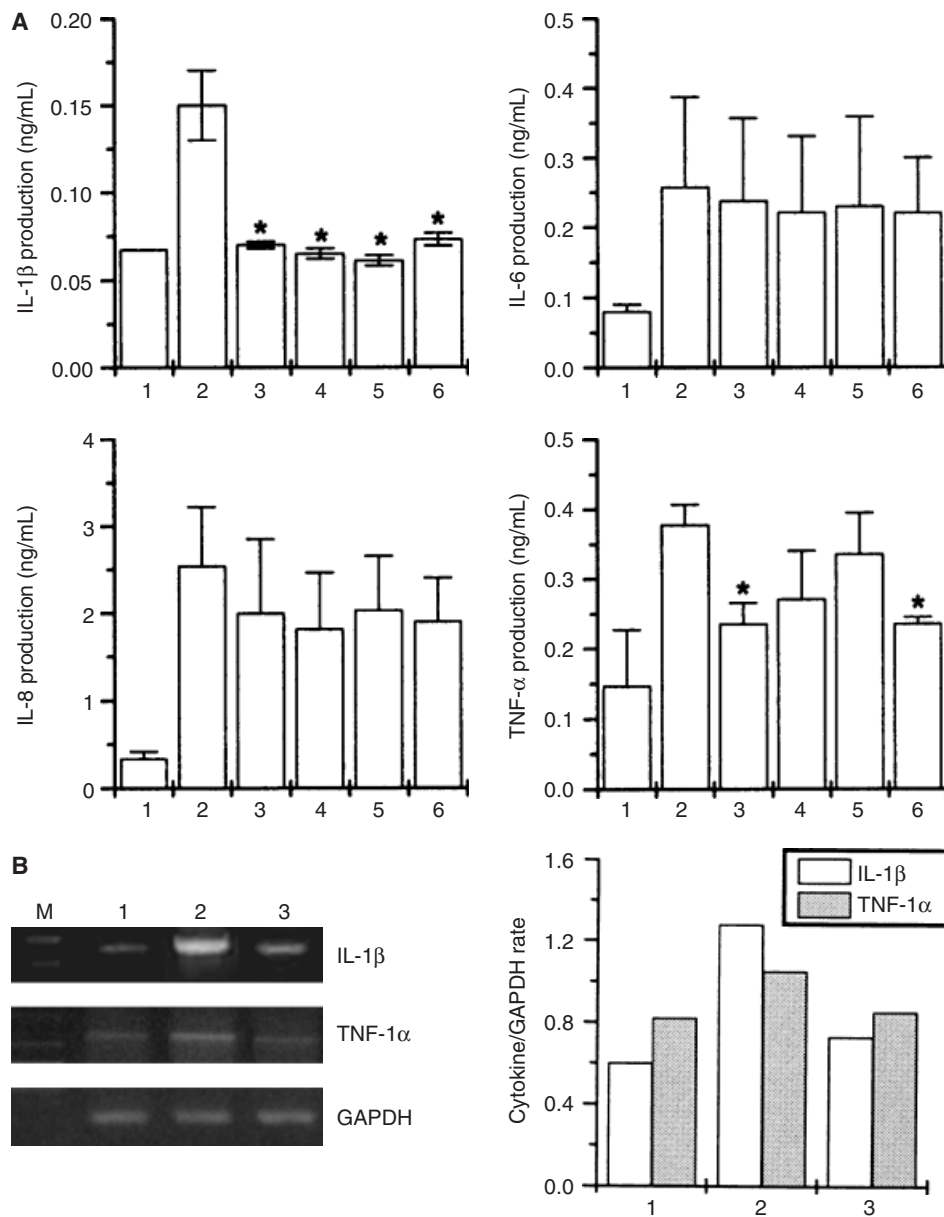


Fig. 4. Effect of alginic acid on phorbol myristate acetate (PMA)+A23187-induced inflammatory cytokine secretion. HMC-1 cells were treated with various concentrations of alginic acid for 1 h and then stimulated with PMA+A23187 for 8 h. Cytokine concentrations were measured from cell supernatants using the ELISA method (a). 1, unstimulated cells; 2, PMA+A23187; 3, PMA+A23187+Alginic acid (0.01 μg/mL); 4, PMA+A23187+Alginic acid (0.1 μg/mL); 5, PMA+A23187+Alginic acid (1 μg/mL); 6, PMA+A23187+DSCG (1 μg/mL). HMC-1 cells were treated with various concentrations of alginic acid for 1 h and then stimulated with PMA+A23187 for 4 h. Messenger RNA was measured using the RT-PCR method (b). The mRNA levels were quantitated by densitometry. M, marker; 1, unstimulated cells; 2, PMA+A23187; 3, PMA+A23187+alginic acid. Values are the mean ± SEM of duplicate determinations from three separate experiments. * $P < 0.05$: significantly different from the PMA+A23187-stimulated cells.

cells, the expression level of NF-κB/*Rel A* increased in the nucleus. However, the expression level of NF-κB/*Rel A* in the nucleus decreased by treatment of alginic acid (Fig. 5a). β-Actin expression level did not change by any treatment in nuclear extract. We next examined whether alginic acid could modulate the luciferase expression specifically via NF-κB. NF-κB luciferase reporter gene constructs (pNF-κB-LUC, plasmid containing NF-κB binding site; Stantagen, La Jolla, CA, USA) were transiently

transfected into HMC-1 cells, which was treated by alginic acid and then stimulated by PMA+A23187. As shown in Fig. 5b, PMA+A23187 increased reporter gene activity. However, the increased activity was decreased by treatment of alginic acid (about 65% inhibition, $P < 0.05$). We also investigated the effect of alginic acid on PMA+A23187-induced NF-κB transcription complex. To perform these studies, we used the NF-κB TF-EIA method. This assay has the advantage of being 10 times more

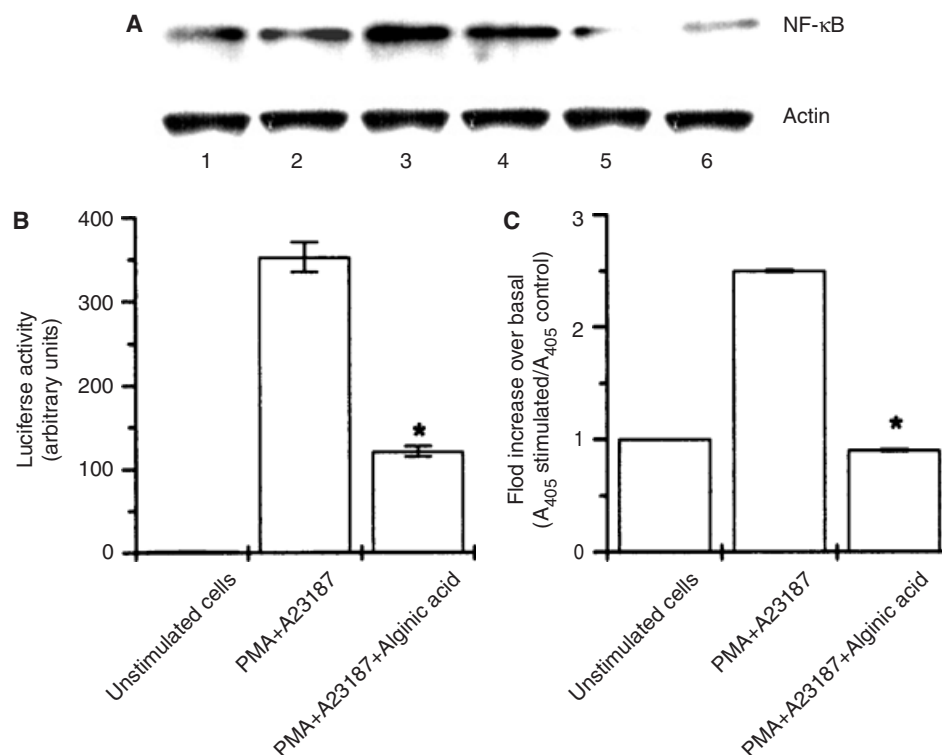


Fig. 5. Effect of alginic acid on the phorbol myristate acetate (PMA)+A23187-induced nuclear factor (NF)- κ B activation. HMC-1 cells were treated with alginic acid (0.01 μ g/mL) for 1 h and then stimulated with PMA+A23187 for 2 h. Nuclear protein was prepared and analysed for NF- κ B by Western blotting as described in the experimental procedures (a). 1–2, unstimulated cells; 3–4, PMA+A23187; 5–6, PMA+A23187+Alginic acid (0.01 μ g/mL). The NF- κ B activity was assayed by luciferase assay (b). Nuclear protein was incubated in a 96-well plate coated with an oligonucleotide containing the NF- κ B-binding site. The presence of an NF- κ B transcription complex was evaluated with an NF- κ B antibody. The result is expressed as the folds' increase of the absorbance at 405 nm over control conditions (c). Values are the mean $\pm$ SEM of duplicate determinations from three separate experiments. * $P < 0.05$: significantly different from the PMA+A23187-stimulated cells.

sensitive than electrophoretic mobility shift assay and allows greater flexibility in the experimental step. As shown in Fig. 5c, PMA+A23187 increased DNA-binding activity for NF- κ B. However, the increased binding activity was decreased by treatment of alginic acid.

Discussion

Alginic acid has long been used for the treatment of various diseases and afflictions/conditions, including cancer, ulcers, and hypotension. Alginic acid oligosaccharide suppresses Th2 development and IgE secretion by inducing IL-12 secretion [16]. Katayama et al. [17] reported that poraprezinc-sodium alginate (P-AG) has a mucosal protective effect, free radical scavenging activity, and tissue repair-promoting action for haemorrhagic erosion and ulcers. Alginic acid is also an agent with a promotive effect on healing of inflammation of gastric and oesophageal mucosa, and radiation stomatitis [18]. Mo et al. [19] reported that alginic acid inhibited the TNF- α -induced intercellular adhesion molecule-1, nitric oxide, and H₂O₂. Asada et al. [20] have reported on an inhibitory effect of alginic acids on hyaluronidase and on histamine

release from mast cells. In the present study, we found that alginic acid inhibited HDC and NF- κ B activation in *in vivo* and *in vitro* models.

Mice were orally administered alginic acid, which inhibited the compound 48/80 or IgE-mediated anaphylactic reaction. Compound 48/80 has been reported to increase the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This report indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells [21]. Hence, it can be hypothesized that alginic acid might act on the lipid bilayer membrane such that it prevents perturbation induced by compound 48/80. DSCG prevents the release of the mediators of type I allergic reaction as a mast cell stabilizer. The drug does not inhibit the binding of IgE to mast cells or the interaction between cell-bound IgE and specific antigen; instead, DSCG suppresses the release of substances (e.g., histamine, SRS-A) [22]. Our results suggest that alginic acid might play a role as a mast cell stabilizer on PCA.

Histamine is an important mediator in anaphylactic reactions, gastric acid secretions, and neurotransmission in the central nervous system. Basophils and mast cells are

the main sources of histamine, which is formed from L-histidine by HDC. Maeda et al. [23] reported that increased HDC activity in HMC-1 induced by PMA or ionomycin is regulated at the translational level. We pre-suppose that alginate acid acts as an inhibitor of translation factors.

Cytokines, such as IL-1, IL-6, IL-8, and TNF- α , are clearly involved in the inflammatory process [24, 25]. Expression of these cytokines is regulated by transcription factors, NF- κ B, AP-1, NF-activated T cells (NFAT), NF-IL6, and hypoxia-inducible factor-1 [11, 26–28]. In our study, alginate acid inhibited IL-1 and TNF- α secretion and mRNA expression but not IL-6 and IL-8. Therefore, we can pre-suppose that alginate acid only acts as a specific inhibitor of transcription factors. Further investigation is necessary to clarify the mechanism of PMA+A23187-induced IL-1 and TNF- α expression by alginate acid.

A transcription factor such as NF- κ B is a candidate for regulation of inflammatory cytokines, IL-1, IL-6, IL-8, and TNF- α gene as consensus-binding sites for the transcription factor are present in that gene [12, 27]. Activation of NF- κ B is dependent on the phosphorylation and degradation of I κ B, an endogenous inhibitor that binds to NF- κ B in the cytoplasm. Previously, glucocorticoids that have frequently been used for the treatment of inflammatory bowel disease and rheumatoid arthritis were suggested to suppress NF- κ B activation. Glucocorticoids are thought to induce transcription of I κ B α , resulting in an enlarged I κ B α pool, and therefore reduced active NF- κ B in the nucleus [29]. Therefore, further investigation is necessary to clarify the inhibition mechanism on PMA+A23187-induced NF- κ B activation by alginate acid. Overall, our findings suggest that alginate acid has various regulatory effects, which might explain its beneficial effect in the treatment of anaphylactic and inflammatory diseases.

Acknowledgements

This research was supported by VestibuloCochlear Research Center of Wonkwang University (R13-2002-055-01003-0).

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