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Achyranthes japonica Nakai Water Extract Suppresses Binding of IgE Antibody to Cell Surface FcεRI.

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ABSTRACT: *Achyranthes japonica* Nakai (AJN) water extract has a variety of physiological properties, including anti-diabetic, anti-cancer, anti-inflammatory, anti-microbial, and anti-oxidative activities. In the present study, the inhibitory effects of AJN extract were investigated in high affinity immunoglobulin E receptor (FcεRI)-mediated KU812F cells activation. AJN extract showed suppressive effects on histamine release and intracellular calcium $[Ca^{2+}]i$ elevation from anti-FcεRI antibody (CRA-1)-stimulated cells in a dose-dependent manner. Flow cytometric analysis showed that AJN extract treatment caused a dose-dependent decrease in the cell surface FcεRI expression and the binding between the cell surface FcεRI and the IgE antibody. Moreover, reverse transcription-polymerase chain reaction analysis showed that levels of the mRNA for the FcεRI α chain was decreased by treatment with AJN extract. These results indicate that AJN extract may exert anti-allergic effects via the inhibition of calcium influx and histamine release, which occurs as a result from the down-regulation of the binding of IgE antibody to cell surface FcεRI. This mechanism may occur through FcεRI expression inhibition.

Keywords: *Achyranthes japonica* Nakai, histamine release, FcεRI, immunoglobulin E, human basophilic KU812F cells

INTRODUCTION

Achyranthes japonica Nakai (AJN) is a perennial herb of the *Achranthes* genus, from the Amaranthaceae family, and it is widely distributed throughout East Asia, including Korea and Japan (1). It has been used as a traditional medicine for the treatment of edema, arthritis, mastitis, and delayed menses (2). This plant contains several important phytochemicals such as saponins, inokosterone, ecdysterone, and oleanolic acid bisdemoside (3,4). Additional biological and pharmaceutical activities of AJN are anti-inflammatory, anti-oxidative, anti-microbial, and osteoprotective activities (1,5-8).

The prevalence and severity of allergic diseases has dramatically increased around the world, especially in developed countries; thus, it is essential that we find preventive strategies to suppress individuals' sensitivities to environmental antigens and the onset of allergic disorders (9). Mast cells and basophils express the high affinity immunoglobulin E receptor, FcεRI, and play an important role in IgE-mediated allergic reaction such as asthma, atopic dermatitis, and food allergy (10). Cross-linking of FcεRI molecules attached to an allergen-specific IgE antibody initiates a cascade of biochemical events that results in elevation of $[Ca^{2+}]i$, and induce the secretion of inflammatory mediators, including histamine, which induces allergic responses, such as asthma, atopic dermatitis, and allergic rhinitis (11-16). FcεRI is a tetrameric receptor composed of one-α, one-β, and two-disulfide linked γ-chains. Among the three subunits of FcεRI, the α-chain is a specific component of FcεRI, expressed on FcεRI-positive cells and mostly extends out into the extracellular region where it binds directly to the Fc portion of the IgE antibody (17). Thus, the down-regulation of FcεRI expression may lead to the inhibition of FcεRI-mediated allergic reactions. Recently, studies concerning anti-allergic effects in FcεRI-mediated allergic reactions reported on extracts of *Chrysanthenum zawadsaki*, *Scutellariae radix*, *Houttuynia cordata* Thunb, and blueberry (18-21). We determined the anti-allergic activities of AJN through inhibition of histamine release in anti-FcεRI antibody (CRA-1)-stimulated KU812F cells. Therefore, in the present study, the suppressive effects of AJN extract on FcεRI-mediated activation of KU812F cells were investigated.

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MATERIALS AND METHODS

Reagents

CRA-1 was purchased from Kyokuto (Tokyo, Japan). Mouse IgG and anti-human IgE fluorescein isothiocyanate (FITC) antibodies were purchased from Biosources (Burlingame, CA, USA). Anti-mouse IgG FITC antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (Baltimore, PO, USA). The RPMI-1640 medium, antibiotics, antimycotics, and fetal bovine serum (FBS) were obtained from GIBCO BRL (Gaithersburg, MD, USA). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was purchased from Promega (Madison, WI, USA). The TRIzol reagent, Superscript II reverse transcriptase, and oligo(dT)$_{12\text{-}18}$ primer were purchased from Invitrogen (Carlsbad, CA, USA). The Taq DNA polymerase was purchased from Roche (Mannheim, Germany). The dNTP set was purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). All other reagents, including hydroxyethyl piperazineethanesulfonic acid (HEPES), Fura 2-acetoxymethyl ester (AM), dimethylsulfoxide, histamine, and *o*-phthalaldehyde (OPA) were purchased from Sigma Chemicals (St. Louis, MO, USA).

Preparation of AJN extract

The dried and powered roots of AJN were immersed in 10 volumes of distilled water and boiled under reflux for 24 h. After centrifugation, the supernatant was filtered, concentrated under vacuum, lyophilized, and stored at −20°C. The lyophilized extract was dissolved in phosphate buffered saline (PBS) and filtered through a 0.45 μm membrane filter before use.

Cell culture, treatment, and determination of cytotoxicity

The KU812F cells, a human basophilic cell line originally isolated from chronic myelocytic leukemia that expresses a FcεRI (22), were provided by Dr. Sanetaka Shirahata, Kyushu University, Fukuoka, Japan. The cells were maintained in an RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 10 mM HEPES, antibiotics, and antimycotics, cultured at 37°C in a humidified atmosphere with 5% CO_2, and passaged every 3∼4 days. Cell viability was measured by the MTS assay using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation (Promega) assay according to the manufacturer's instructions. The KU812F cells were seeded on 96-well plates at a density of 2.5×10^4 cells/well, and incubated with serum-free medium in the presence of various concentrations of AJN for 24 h. The culture medium was removed and replaced with 95 μL of fresh culture medium and 5 μL of MTS solution. The cells were incubated for 1 h, and the absorbance was measured at 490 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Relative cell viability was calculated and compared with the absorbance seen in the untreated cells.

Histamine assay

The histamine content was assessed using a spectrofluorometric assay (23). The KU812F cells (1×10^6 cells/mL) were treated with different concentrations of AJN extract (10, 20, 50, and 100 μg/mL) for 24 h. The treated cells were suspended in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, and 1.8 mM $CaCl_2$) and stimulated with CRA-1 (10 μg/mL) for 30 min at 37°C. After centrifugation, the supernatant (100 μL) was collected and 40 μL of 1 N NaOH and 20 μL of 0.2% OPA were added. The mixture was incubated on ice for 40 min, and the reaction was terminated by adding 10 μL of 3 N HCl. The fluorescence intensity was measured using a FLx800 microplate fluorescence reader (BioTek Instruments Inc., Winooski, VT, USA) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

Measurement of intracellular Ca^{2+}

The intracellular Ca^{2+} concentration was measured using a calcium reactive fluorescence probe, Fura 2-AM. The KU812F cells (1×10^6 cells/mL) were treated with various concentration of AJN extract, harvested, and suspended in 100 μL of Tyrode's buffer containing 2.0 μM of Fura 2-AM, and incubated at 37°C for 30 min. The cells were then washed three times with PBS and stimulated with 100 μL of 10 μg/mL CRA-1. The Fura 2 fluorescence was monitored with a microplate fluorescence reader at an excitation wavelength of 360 nm and an emission wavelength of 528 nm.

Flow cytometry analysis

The cell surface expression of FcεRI was measured with indirect immunofluorescence and flow cytometry. Briefly, KU812F cells (1×10^6 cells/mL) were treated with various concentrations of AJN extract for 24 h, and washed twice with PBS. The cells were incubated with 100 μL of 10 μg/mL CRA-1 on ice for 60 min and washed three times with ice-cold PBS. The cells were then stained with 100 μL of 20 μg/mL anti-mouse IgG FITC antibody on ice for 60 min, followed by washing three times with 1 mL of ice-cold PBS for flow cytometric analysis (EPICS® XL, Beckman Coulter, Inc., Fullerton, CA, USA). As a negative control, 100 μL of 10 μg/mL mouse IgG antibody was used instead of CRA-1. The anti-human IgE FITC antibody was used to measure the binding between IgE and FcεRI. The percentage of FcεRI-positive cells was calculated with an arbitrary cutoff of 2%, as determined by the negative control.

Reverse transcription-polymerase chain reaction (RT-PCR)

The FcεRI α chain mRNA levels were determined by RT-PCR. Briefly, KU812F cells (1×10^6 cells/mL) were treated with various concentrations of AJN extract for 24 h, were harvested by centrifugation, and the pellet was then washed twice with PBS. Total cellular RNA was isolated using 1 mL of TRIzol RNA extraction reagent, according to the manufacturer's instructions. For cDNA synthesis, 0.5 μg/mL of an oligo$(dT)_{12\text{-}18}$ primer and RNA free water were added to 1 μg of total RNA. And then this mixture was denatured at 70°C for 5 min and cooled immediately. The RNA was reversed transcribed in a master mix containing 4 μL of 5× first strand buffer, 2 μL of 0.1 M dithiothreitol, 1 μL of 10 mM dNTP and, and 1 μL of Superscript II reverse transcriptase at 42°C for 50 min and at 70°C for 15 min. One μL of resultant cDNA samples was subjected to PCR amplification in the presence of specific sense and antisense primers. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as the internal control. The primer sequences were as follows: FcεRI α-chain, sense 5'-CTT AGG ATG TGG GTT CAG AAG T-3' and antisense 5'-GAC AGT GGA GAA TAC AAA TGT CA-3'; G3PDH, sense 5'-GCT CAG ACA CCA TGG GGA AGG T-3' and antisense 5'-GTG GTG CAG GAG GCA TTG CTG A-3'. The PCR was conducted as follows: 94°C, 30 s denaturing; 55°C, 30 s annealing; and 72°C, 1 min extension. The cDNA was subjected to 18 and 23 cycles for FcεRI α chain and G3PDH genes, respectively. The amplified PCR products were visualized with 1% agarose gel electrophoresis and ethidium bromide staining, then analyzed using a Molecular Imager® Gel Doc™ XR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

All measurements were conducted independently, at least in triplicate. The data are expressed as the mean±standard deviation (SD). The statistical differences between the control and the AJN extract groups were determined by the Student's *t*-test, and the results were considered statistically significant at $P\leq0.01$.

RESULTS AND DISCUSSION

Effects of AJN extract on cell viability

AJN has been used for the treatment of edema, arthritis, mastitis, and delayed menses, and it has been shown to exert various physiological activities including anti-inflammatory, anti-oxidative, anti-microbial, and osteoprotective activities (1,5-8). The AJN water extract was examined for its inhibitory effects on FcεRI-mediated KU812F cells activation and may be an important biologically active anti-allergic material. Prior to treatment with AJN extract, cytotoxicity levels were assessed. The cells were cultured in serum-free RPMI-1640 medium with AJN extract (0, 10, 50, and 100 μg/mL) and assessed by the MTS assay. The AJN extract showed no cytotoxic effects at a concentration of 100 μg/mL (Fig. 1).

AJN effects on FcεRI-mediated histamine release

The activation of basophils and mast cells through FcεRI is triggered by aggregation of FcεRI, bound to allergen specific IgE on the cell surface, and degranulation, resulting in a release of mediators (12,13,24). Histamine, which is stored in the secretory granules, is released in immunologically activated mast cells and basophils (11). Thus, the histamine in the medium was used as a marker of the degranulation of mast cells and basophils (25). In order to assess AJN's inhibitory effects on degranulation, KU812F cells were treated with AJN extract, and stimulated with CRA-1. The amount of histamine re-

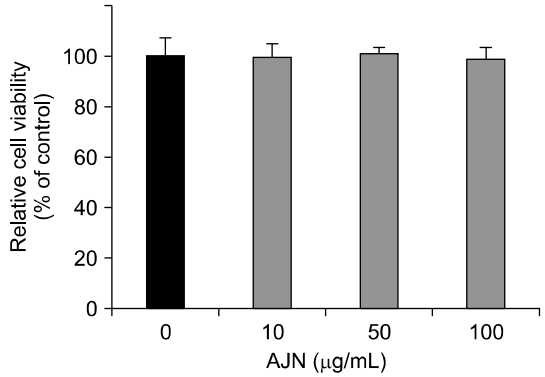

Fig. 1. Effects of *Achyranthes japonica* Nakai (AJN) extract on cell viability. KU812F cells were cultured in the presence of AJN extract (0, 10, 50, and 100 μg/mL) for 24 h under serum-free conditions. The cell viability of KU812F cells was determined by the MTS assay. Each value represents the mean±SD of three independent experiments.

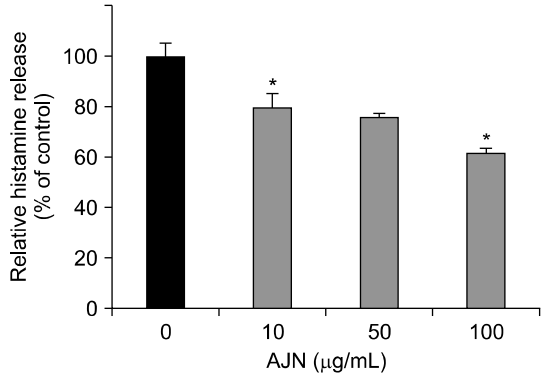

Fig. 2. Effects on FcεRI-mediated histamine release. Treated cells were stimulated with anti-FcεRI antibody-1. Histamine released from the stimulated cells was measured by spectrofluorometric analysis using OPA. Each value represents the mean±SD of three independent experiments. *Values are significantly different from control ($P<0.01$). AJN, *Achyranthes japonica* Nakai.

leased from the cells was spectrofluorometrically determined using OPA. As shown in Fig. 2, the histamine released from CRA-1-stimulated cells was 80.2%, 75.6%, and 62% when the cells were treated with 10 μg/mL, 50 μg/mL, and 100 μg/mL of extract, respectively. This evidence suggests that AJN extract could be useful for the prevention of FcεRI-mediated degranulation of KU812F cells.

AJN effects on $[Ca^{2+}]i$ elevation

An increase in the intracellular concentration of Ca^{2+} is essential for mast cell degranulation and cytokine responses (11). To gain information regarding the inhibitory mechanism of FcεRI-mediated activation by AJN extract, intracellular calcium concentration was spectrofluorometrically examined in CRA-1-stimulated KU812F cells using a calcium specific fluorescent probe, Fura 2-AM. In the AJN extract-treated cells, the $[Ca^{2+}]i$ was reduced in a dose-dependent manner (Fig. 3). This result indicates that AJN extract suppression of $[Ca^{2+}]i$ elevation may be associated with degranulation of mast cells and basophils. We demonstrated that AJN extract treatment dose-dependently inhibited FcεRI-mediated $[Ca^{2+}]i$ elevation.

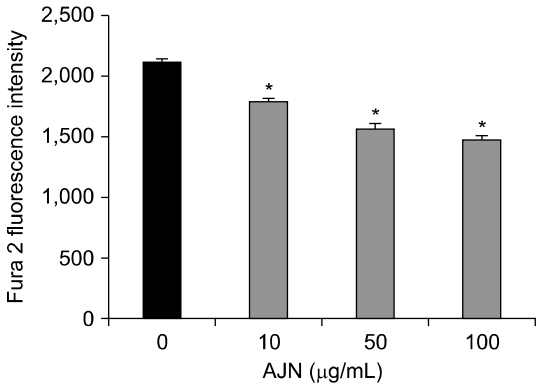

Fig. 3. Effect on FcεRI-mediated intracellular calcium elevation. Pretreated cells were stimulated and treated with Fura 2-acetoxymethyl ester. The fluorescence intensity was measured. Each value represents the mean±SD of three independent experiments. *Values are significantly different from control ($P<0.01$). AJN, *Achyranthes japonica* Nakai.

The FcεRI cross-linking activates downstream signaling cascades including the activation of protein tyrosine kinases such as Syk, Lyn, calcium mobilization, and protein kinase C translocation required for degranulation (26,27). Therefore, further studies mechanism of FcεRI-mediated signaling events by AJN extract are needed.

AJN effects on FcεRI α chain cell surface expression

The FcεRI is a high-affinity IgE receptor. Among the FcεRI subunits, the α chain extends into the extracellular region of FcεRI and binds to the Fc portion of IgE, with high affinity (18,28,29).

To assess the inhibitory effects on cell surface FcεRI expression, KU812F cells were treated with different concentrations of AJN extract for 24 h under serum-free conditions, and the cell surface FcεRI expression was

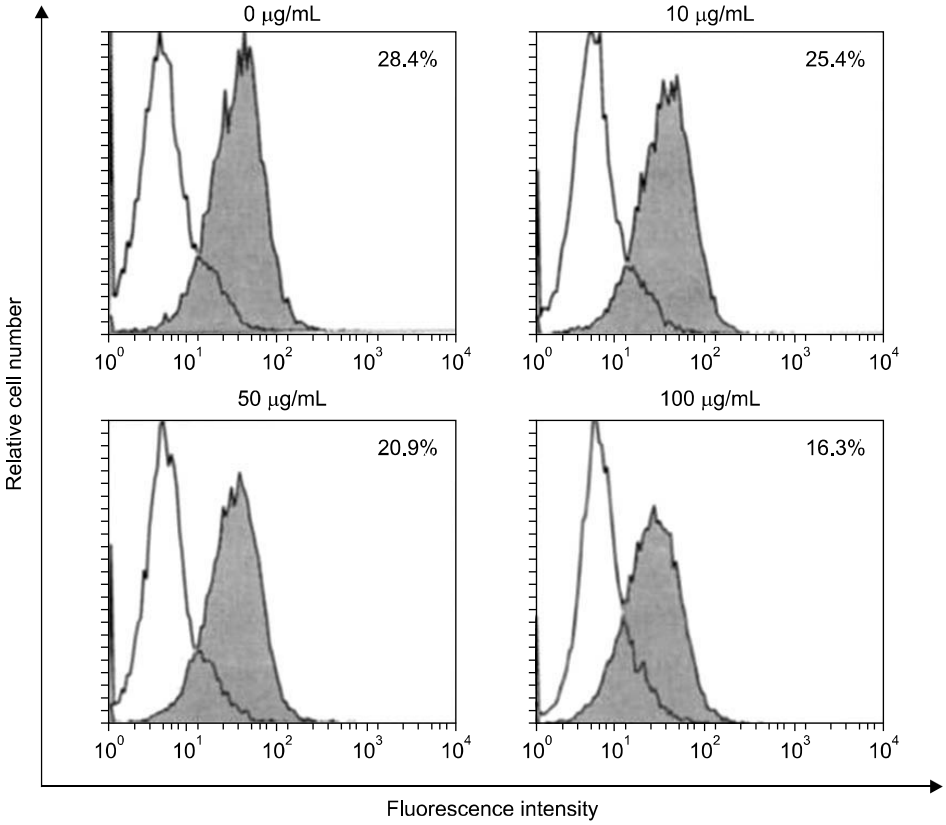

Fig. 4. Effects on the cell surface FcεRI α chain expression. Treated cells were incubated with anti-FcεRI antibody-1, stained, and analyzed by flow cytometry.

evaluated by flow cytometry using CRA-1. Cell surface FcεRI expression was reduced from 28.4, 25.4, 20.9, and 16.3% after AJN extract treatment at concentrations of 10, 50, and 100 μg/mL, respectively (Fig. 4).

AJN effects on FcεRI α chain mRNA expression

To further analyze the inhibitory effects of AJN extract on FcεRI α chain gene expression, mRNA levels were measured by RT-PCR. The FcεRI α chain mRNA level of non-treated cells was clearly detected; however, the corresponding mRNA level of AJN extract-treated cells was reduced in a dose-dependent manner (Fig. 5). These results show that the AJN extract-induced reduction in FcεRI α chain mRNA levels may be associated with the suppression of cell surface expression causing degranulation inhibition of basophils via down-regulation of calcium influx and FcεRI expression. This mechanism contributes to the prevention and treatment of allergic reactions.

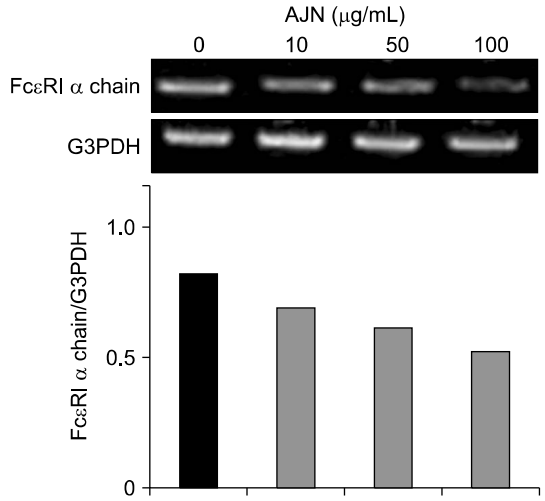

Fig. 5. Effects on the FcεRI α chain mRNA level. Total cellular RNA was isolated from prepared cells, and the FcεRI α chain mRNA level was analyzed by RT-PCR. G3PDH, human glyceraldehyde-3-phosphate dehydrogenase.

AJN extract suppresses binding of IgE antibody to cell surface FcεRI

The binding of IgE to cell surface FcεRI was assessed by flow cytometry using human IgE antibody. As shown in Fig. 6, the binding activity was observed to be 26.8, 24.7, 21.1, and 15.9% for the AJN extract treatment levels of 0, 10, 50, and 100 μg/mL, respectively. Basophils and mast cells are sensitized after binding IgE with cell surface FcεRI. Basophils and mast cells degranulate by cross-linking the cell surface FcεRI with IgE molecules and allergens (13,16,17). The binding of IgE antibody to cell surface FcεRI was found to be down-regulated by the AJN extract in a dose-dependent manner (Fig. 6).

Considering the role of FcεRI in IgE-mediated allergic reactions, inhibition of allergen-IgE-FcεRI formation by AJN extract may also be useful in allergic disease prevention. These results suggest that AJN extract might negatively regulate the activation of basophils through the suppression of FcεRI expression and thereby contrib-

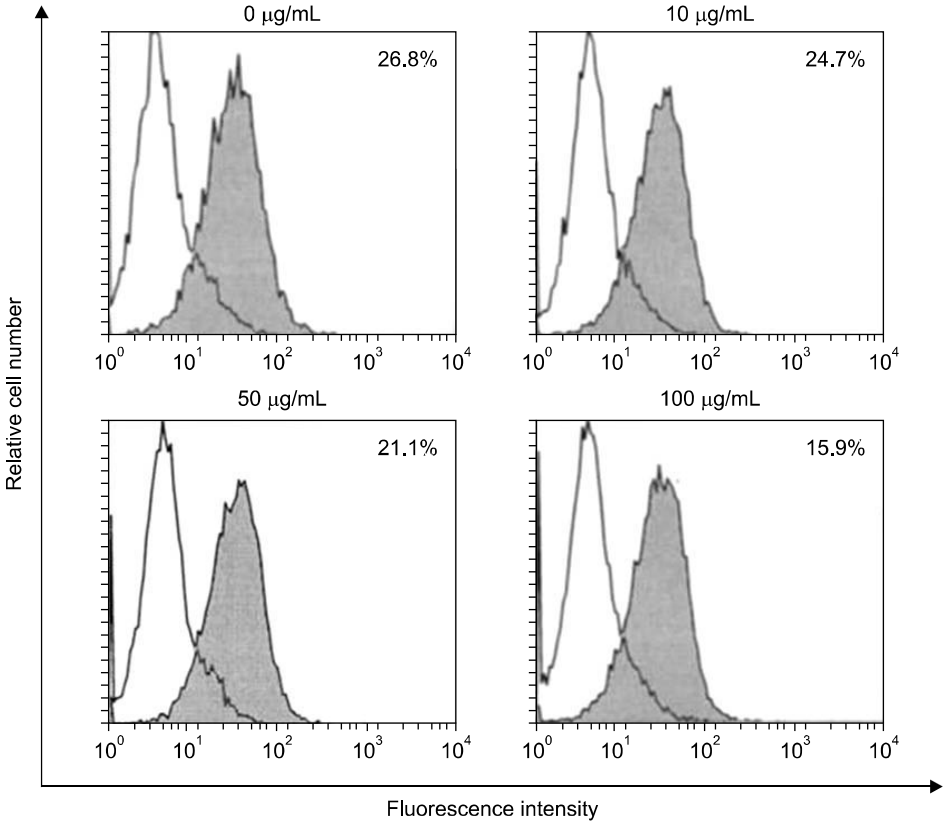

Fig. 6. *Achyranthes japonica* Nakai extract suppresses binding of IgE antibody to cell surface FcεRI. Treated cells were incubated with human IgE antibody, stained, and analyzed by flow cytometry.

ute to the decrease of allergic reactions. AJN contains several biological and pharmaceutical components such as saponins, inokosterone, ecdysterone, and oleanolic acid bisdemoside (2-4). Recently, many studies have shown the anti-allergic effects of bioactive compounds such as catechin and (−)-epigallocatechin-3-*O*-gallate from green tea, kaempferol from *Nelumbo nucifera* stamens, and phlorotannins from *Ecklonia stolonifera* on FcεRI-mediated allergic reactions (30-34). However, the down-regulation of FcεRI expression by bioactive compounds from AJN in basophils and mast cells has not been studied. These results suggest that AJN may play a role in the prevention and treatment of FcεRI-mediated allergic conditions. Further studies on the bioactive components of AJN are necessary to elucidate the suppressive mechanism of FcεRI-mediated allergic reactions.

Our findings provide the first experimental evidence that AJN extract suppresses FcεRI expression. These results indicate that AJN extract may exert an anti-allergic effect by inhibiting calcium influx and histamine release from the down-regulation of cell surface FcεRI and the IgE antibody binding through the inhibition of FcεRI expression.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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