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Inhibitors for expression of IgE receptor on human mast cell from Puerariae Flos

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ABSTRACT

Bioassay-guided separation of the extract of the medicinal plant, Puerariae Flos, disclosed the two isoflavones tectorigenin (**1**) and genistein (**2**) as the inhibitors for expression of IgE receptor (FcεRI), the key molecule triggering the allergic reactions, on human mast cells. As a result of analysis of structure–activity relationship of the naturally occurring and synthesized isoflavones, 7-*O*-methyl glycitein (**11**) was disclosed as the more potent inhibitor than tectorigenin (**1**). These isoflavone ingredients suppressed expression of FcεRI more potently than the active flavonoids found previously. In addition, tectorigenin (**1**) was clarified to particularly reduce generation of γ-chain subunit to suppress expression of FcεRI among the three subunits.

The high-affinity receptor of IgE (FcεRI), comprised of one α-chain, one β-chain, and two disulfide-linked γ-chain, is the key molecule for triggering IgE-mediated allergy reactions. Multivalent allergens bridge the receptor-bound IgE to induce aggregation of FcεRI on the surface of mast cells. This sequential biological response after expression of FcεRI results in secretion of the allergic mediators, histamine, proteases, chemotactic factors, and arachidonic acid metabolites, as well as sequential transcription of the cytokine genes responsible for allergic symptoms.[1] Furthermore, the FcεRI-deficient mice, generated by disruption of the gene due to α-chain of FcεRI, were shown to be resistant to anaphylaxis and survive normally.[2] Thus, suppression for FcεRI expression on mast cells is considered as an attractive target for preventing the IgE-mediated allergic symptoms free from any harmful side effects. In spite of significant expectation for anti-allergic seed principles with a new mechanism of action, small-molecule inhibitors for FcεRI expression on human mast cells have been little revealed.

In this context, we have been engaged in search for anti-allergic scaffolds with inhibitory activity for expression of FcεRI. Previously, we disclosed the flavonoids bearing a pyrogallol function as active principles.[3] Further exploration of new active principles from medicinal plants led us to isolate the two isoflavones, tectorigenin (**1**) and genistein (**2**) from Puerariae Flos. Herein, we wish to describe the bioassay-guided isolation, identification, and biological property but also comparative analysis of the mechanism of action between these isoflavones and the precedented inhibitors. Moreover, we deal with structure–activity relationship in the isoflavones involving the synthesized analog with more potent activity than isolated **1**.

In the bioassay to explore active principles, inhibitory potency was evaluated by amount of IgE receptors on surfaces of human mast cells determined by indirect fluorescent antibody technique using flow cytometry.[4] In brief, HMC-1 cells,[5] the human mast cell line established by Butterfield et al., were incubated in the presence of tested samples for 72 h, thereafter the harvested cells were treated with anti-FcεRI α-chain antibody followed by FITC-labeled antibody. After this treatment, IgE receptors were detected as fluorescence of FITC with a flow cytometer. By use of this assay for the extracts from about 400 medicinal plants, the MeOH extract of Puerariae Flos (flowers of *Pueraria thomsonii* Benth.) was shown to intensively inhibit expression of FcεRI on human mast cells. After this extract was successively partitioned between EtOAc and H_2O, *n*-BuOH and H_2O, the resulting EtOAc extract exhibited the most potent activity among the three extracts. Bioassay-guided separation of the EtOAc extract through SiO_2 and ODS column chromatography followed by normal and reversed phase HPLC resulted in the isolation of two active principles **1** and **2** in 0.24% and 0.044% yield from the crude drug, respectively.

The molecular formula $C_{16}H_{12}O_6$ of **1** was determined by FAB-HRMS ($[M+H]^+$ *m/z* 301.0715, calcd 301.0712) implying eleven degrees of unsaturation. The IR absorptions at 3455, 1678, and 1622 cm^{-1} indicated the presence of the hydroxyl and conjugated enone functionalities. The ^1H NMR spectrum of **1** showed the signals due to the hydroxyl group forming the hydrogen bond [δ_H 13.2 (OH, 1H, br s)], the pentasubstituted aromatic ring [δ_H 6.51 (H-8, 1H, s)], the 1,4-disubstituted aromatic ring [δ_H 7.46 (H-2′,6′,

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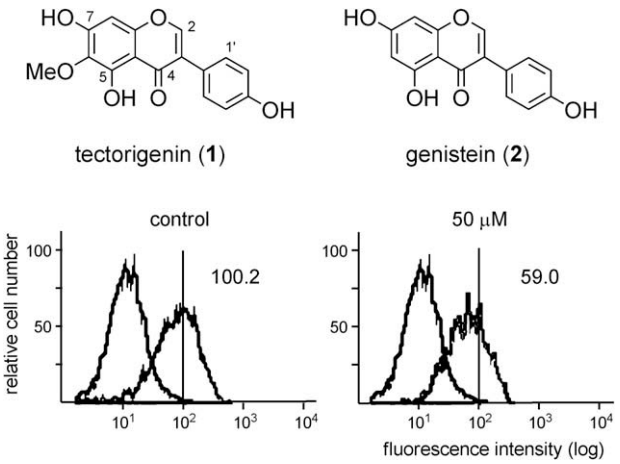


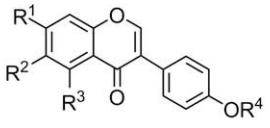
Figure 1. Inhibitors for FcεRI expression on human mast cells from Puerariae Flos. The representative histogram by treatment with tectorigenin (**1**) is illustrated. After exposure of HMC-1 cells to **1** for 72 h, the harvested cells were analyzed by flow cytometry. The left histograms indicate fluorescence intensity due to only treatment with FITC labeled anti-mouse IgG antibody as negative controls, while the expressed FcεRI in the presence or absence of tectorigenin (**1**) is depicted by the right histograms. The vertical line designates the mean of fluorescence intensity without addition of **1**. The value corresponds to the mean of fluorescence intensity in each treatment.

2H, d, J = 8.5 Hz), 6.91 (H-3′,5′, 2H, d, J = 8.5 Hz)], and the α,β-unsaturated enone moiety [δ_H 8.18 (H-2, 1H, s)] together with the methoxyl signal [δ_H 3.89 (3H, s)]. Furthermore, the ^{13}C NMR spectrum of **1** exhibited one carbonyl carbon [δ_C 182.0 (C-4)] and six sp^2 carbons bearing oxygen functions [δ_C 158.5 (C-4′), 157.5 (C-7), 154.6 (C-2), 154.4 (C-8a), 154.2 (C-5), 132.4 (C-6)]. Taking these physicochemical properties into account, the active principle was unambiguously identified as the isoflavone, tectorigenin (**1**), by comparison of the reported spectroscopic data.[6] The other active ingredient, possessing the molecular formula of $C_{15}H_{10}O_5$ ([M+H]$^+$ m/z 271.0610, calcd 271.0606), exhibited the similar NMR spectrum to **1**. However, the signal ascribable to a pair of meta-coupled aromatic protons [δ_H 6.41, 6.29 (H-6,8, 1H each, both d, J = 2.1 Hz)] newly appeared instead of the aromatic proton (H-8) and methoxyl signal of **1** in the NMR spectrum of **2**. Accordingly, the active ingredient was definitely identified as genistein (**2**) in comparison of the physicochemical properties with those in the literature.[7] As shown in Figure 1, the average of fluorescence due to FcεRI is apparently reduced by treatment with tectorigenin (**1**).

Because of wide distribution of isoflavones in natural plant resources including medicinal plants, we next evaluated several commercially available isoflavones for inhibitory potency for FcεRI expression on human mast cells to search congenic inhibitors superior to **1**. Figure 2 summarizes the biological scores of the tested six ingredients along with the isolated tectorigenin (**1**) and genistein (**2**) from Puerariae Flos. Among the tested ingredients, daidzein (**3**) and glycitin (**8**) suppressed expression of FcεRI on HMC-1 comparable to **1**. Interestingly, only glycitein (**7**) exhibited more potent suppressive activity at a concentration of less than 25 μM as compared with tectorigenin (**1**). In respective comparison of biological activity between **3** and **4**, **7** and **8**, introduction of the glucosyl residue to the 7-OH group attenuates the inhibitory potency. As can be seen from the biological outcome of **2** and **6**, the 5,7-dihydroxy function brings about cytotoxicity against HMC-1 in preference to receptor suppressive activity. By comparing the activity in the three pairs of relatives (**1** and **7**, **2** and **3**, **5** and **6**) at a concentration of 5 μM, the hydroxyl group at C-6 is inclined to suppress expression of FcεRI. Comparison of the activity in the three pairs (**1** and **2**, **3** and **7**, **4** and **8**) at a concentration of 5 μM suggests the tendency that the methoxyl function at C-7 enhances the inhibitory effect.

To search more potent congeners free from cytotoxicity than tectorigenin (**1**) as well as examine influence of the substituents at C-6 and C-7 on the inhibitory activity of FcεRI expression, we synthesized the demethyl, the isomethyl, and the 7-*O*-methyl analogs (**9**-**11**) of glycitein (**7**) and evaluated their biological property. The synthesis of the three analogs was conducted as shown in Scheme 1. After introduction of the acetyl group into sesamol (**12**) with acetic anhydride by $BF_3{\cdot}OEt_2$ catalyzed Friedel–Crafts acylation, removal of the methylene bridge over the pyrocatechol moiety by BBr_3 afforded triol **13** in 92% yield for two steps. All of the three hydroxyl groups in **13** were benzylated by treatment with benzyl bromide and K_2CO_3 to give **14a** in 72% yield. Successive methylation of the phenolic hydroxyl groups by trimethylsilyl diazomethane in the presence of diisopropylethylamine and MeOH selectively provided both monomethyl and dimethyl ethers,[8] which was respectively subjected to benzylation under the same condition for preparing **14a** to afford **14b** and **14c** in 79% and 88% yield for two steps. Aldol condensation of the methyl ketones **14a**–**c** with 4-benzyloxybenzaldehyde (**15**) by KOH gave the corresponding chalcones **16a**–**c** in 90%, 85%, and 81% yield, respectively.

Hypervalent iodine mediated rearrangement of the 4-benzyloxyphenyl residue followed by nucleophilic addition of the methoxyl groups proceeded disagreeably to provide dimethyl acetal in



	R^1	R^2	R^3	R^4
tectorigenin (**1**)	OH	OMe	OH	H
genistein (**2**)	OH	H	OH	H
daidzein (**3**)	OH	H	H	H
daidzin (**4**)	OGlc	H	H	H
formononetin (**5**)	OH	H	H	Me
biochanin (**6**)	OH	H	OH	Me
glycitein (**7**)	OH	OMe	H	H
glycitin (**8**)	OGlc	OMe	H	H

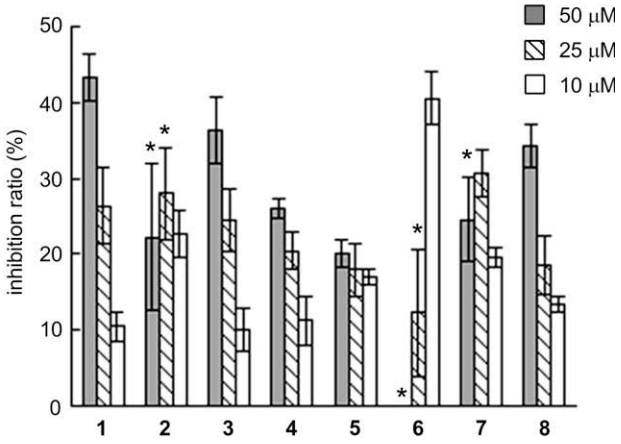
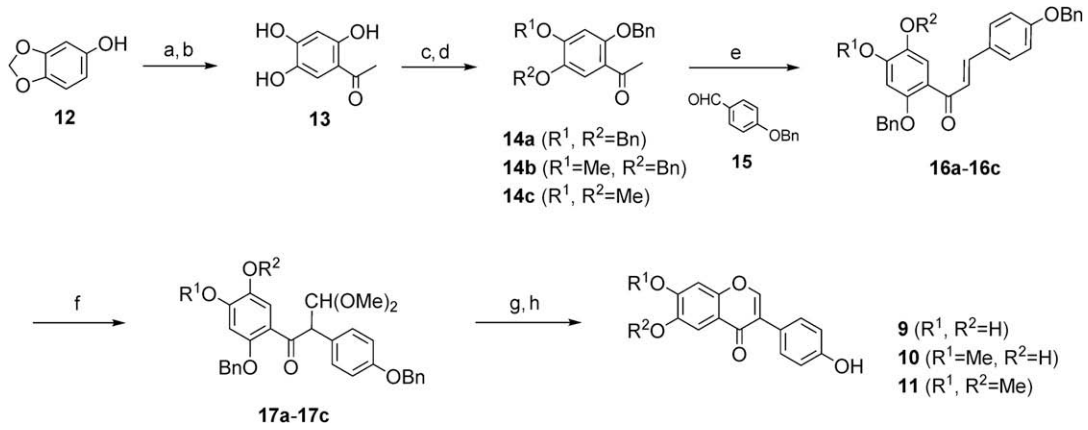


Figure 2. Inhibitory activity of naturally occurring isoflavones for FcεRI expression. *Cytotoxicity was detected by the cytogram in FACS analysis.



Scheme 1. Synthesis of glycitein analogs **9**–**11**. Reagents and conditions: (a) $BF_3{\cdot}Et_2O$, Ac_2O, 80 °C; (b) BBr_3, CH_2Cl_2, rt, 92%, two steps; (c) $TMSCHN_2$, *i*-Pr_2NEt, CH_3CN, MeOH, rt; (d) BnBr, K_2CO_3, acetone, 50 °C, 72% for **14a**, 79% for **14b**, 88% for **14c**, two steps; (e) **15**, KOH, MeOH–THF, reflux, 90% for **16a**, 85% for **16b**, 81% for **16c**; (f) PIDA, $TsOH{\cdot}H_2O$, CH_2Cl_2–$CH(OMe)_3$–MeOH, rt; (g) 5% Pd–C, H_2, MeOH–acetone, rt; (h) concd HCl, MeOH, reflux, 89% for **9**, 89% for **10**, 96% for **11**, three steps.

20% yield[9] under the reaction conditions [phenyliodine bis(trifluoroacetate) (PIFA) and TFA in trimethyl orthoformate] described in the literature.[10] On the contrary, treatment of **16a** with phenyliodine diacetate (PIDA) and $TsOH{\cdot}H_2O$ in CH_2Cl_2–MeOH–$CH(OMe)_3$ proceeded favorably to afford the dimethylacetal **17a** in 89% yield.[9] Removal of the benzyl protection by Pd–C under a H_2 atmosphere and the sequential intramolecular transacetalization and elimination of MeOH furnished the envisioned glycitein analogs (**9**, **10**, and **11**)[11] in 89%, 89% and 96% yield for three steps, respectively.

With regard to the synthesized analogs **9**-**11**, inhibitory activity of FcεRI expression on HMC-1 was assessed and the result is illustrated in Figure 3. Among the three synthesized analogs, 7-*O*-methyl analog **11** showed the strongest activity. Notably, the analog **11** inhibited expression of FcεRI more potently than tectorigenin (**1**) without showing any cytotoxicity even at a concentration of 50 μM different from glycitein (**7**). On the demethyl analog **9** possessing the pyrocatechol moiety, the biological potency was found to be considerably declined. Furthermore, the two analogs except for **11** lack concentration dependency for the receptor suppressing activity.

FcεRI is the tetrameric receptor consisting of one α-chain, one β-chain, and two disulfide-linked γ-chains. Besides this αβγ2 tetramer, another structure, αγ2 trimer, is also found in humans. Previously, we clarified the active flavonoids containing the pyrogallol moiety as B-ring to induce down-regulation for γ-subunit specifically. Therefore, we analyzed the subunit responsible for down-regulation of FcεRI expression due to the isoflavone, tectorigenin (**1**), by monitoring generation of mRNA.[12] HMC-1 cells were cultured in the presence of **1** for 36 h, and total mRNA was collected by use of oligo (dT)-cellulose column in the usual manner. Each mRNA level of α-, β- and γ-chain was determined by RT-PCR followed by staining the provided PCR product with ethidium bromide. As shown in Figure 4, tectorigenin (**1**) apparently suppressed mRNA expression of γ-chain, while no obvious alternation was observed with respect to mRNA expression of α- and β-chains. Therefore, tectorigenin (**1**) was clarified to principally reduce generation of γ-chain subunit to suppress expression of FcεRI.

In conclusion, bioassay-guided separation of the extract of the medicinal plant, Puerariae Flos, revealed the two isoflavones, tectorigenin (**1**) and genistein (**2**), as the inhibitors for FcεRI expression on human mast cells, which would be recognized as promising seeds toward anti-allergic agents with the new mechanism of action. As a result of analysis of structure–activity relationship of the naturally occurring and synthesized isoflavones, 7-*O*-methyl glycitein (**11**) was disclosed as the more potent inhibitor than tectorigenin (**1**). Furthermore, the isoflavones were shown to predominantly reduce generation of γ-chain subunit to suppress expression of FcεRI among the three subunits. It should be noted that these isoflavone ingredients suppressed expression of FcεRI more potently than the active flavonoids previously found by our group. In addition, the present inhibitors are also distinguished from the precedents by the structural features, different special placement of B-ring to A- and C-rings as well as the absence of the pyrogallol function.

To date, no anti-allergic agents based on suppression of FcεRI expression have been developed. Although the present active ingredients may require a little high concentration for this biological response in practice, tectorigenin (**1**) has been revealed to be the most potent suppressor as a result of exhaustive survey of the screened about 400 medicinal plants. However, the potential of the active constituent described here for the anti-allergic agents

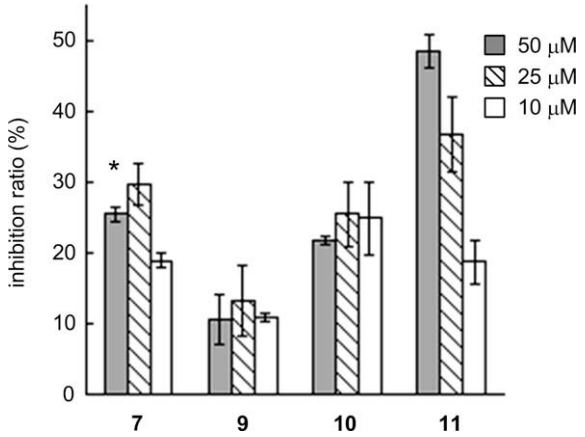


Figure 3. Inhibitory activity of glycitein analogs for FcεRI expression. *Cytotoxicity was detected by the cytogram in FACS analysis.

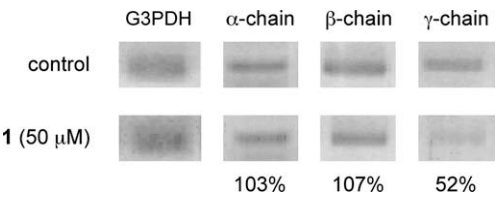


Figure 4. Inhibition for expression of mRNA of FcεRI subunits by tectorigenin (**1**). Relative ratio of expression of each subunit by treatment with tectorigenin (**1**) is listed by percentage.

with the novel mechanism of action is unconcluded at present. To open up a new avenue to this issue, further investigations including human clinical trial and development of sensitive bioassay should be regarded to be crucial.

Acknowledgments

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4. In 24-well microculture plates, HMC-1 cells (5.0×10^5 cells/mL) were cultured in 0.98 mL of IMDM medium (Sigma) containing 10% fetal bovine serum (Sigma), 2% L-Glutamine Stock solution (Nacalai), 1.2% Penicillin–Streptomycin-solution (Sigma), and 1.2 mM monothioglycerol (Sigma) in the presence of the test samples at 37 °C in 5% CO_2 for 72 h. The test samples were dissolved in DMF and diluted to the appropriate concentration using complete medium, then 20 μL of each sample solution was inoculated. The final concentration of DMF in the culture is 0.2%. After the whole was washed with PBS containing 0.5% BSA and 0.05% NaN_3 twice, the cells were treated with anti-human FcεRI α-chain antibody (0.001 μg, Kyokuto) on ice for 60 min. Then the cells were rinsed with the PBS twice and incubated with FITC labeled anti-mouse IgG antibody (0.001 μg, Cosmo Bio) on ice for 45 min in the dark. After duplicate washing with the PBS, the harvested cells were analyzed with a flow cytometer (FACScalibur, Becton Dickinson). All samples were assessed for inhibitory activity for FcεRI expression in triplicate. In the cases of co-incubation with only DMF and treatment with only FITC labeled anti-mouse IgG antibody, the fluorescent scores were indicated as mean (control) and mean (back), respectively. Inhibition ratio of each sample was determined by the following equation: Inhibition ratio (%) = 100 × [mean (control) − mean (sample)/mean (control) − mean (back)].
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9. Since the dimethyl acetals **17a**–**17c** could not purified by SiO_2 column chromatography because of considerable lability, the yields from the chalcones **16a**–**16c** to the glycitein analogs **9**–**11** are listed. On the basis of observation on the TLC profiles of the reactions from **17a**–**17c** to **9**–**11**, the two conversions were discriminated to proceed quantitatively. Thus, the yields may be considered in the transformation of **16a**–**16c** into **17a**–**17c**.
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11. Compound **9**: a pale yellow powder. ^{1}H NMR (300 MHz, acetone-d_6) δ: 8.22 (1H, s, 2-H), 7.36 (1H, s, 5-H), 7.34 (2H, d, J = 8.6 Hz, 2′,6′-H), 6.84 (1H, s, 8-H), 6.78 (2H, d, J = 8.6 Hz, 3′,5′-H). FAB-MS (m/z): 271 $[M+H]^+$. HR FAB-MS (m/z): calcd for $C_{15}H_{10}O_5$+H: 271.2369, found: 271.2364. **10**: a pale yellow powder. ^{1}H NMR (300 MHz, acetone-d_6) δ: 8.33 (1H, s, 2-H), 7.38 (1H, s, 5-H), 7.39 (2H, d, J = 8.6 Hz, 2′,6′-H), 7.16 (1H, s, 8-H), 6.80 (2H, d, J = 8.6 Hz, 3′,5′-H), 3.91 (3H, s, OCH_3). FAB-MS (m/z): 285 $[M+H]^+$. HR FAB-MS (m/z): calcd for $C_{16}H_{12}O_5$+H: 285.2635, found: 285.2634. **11**: a pale yellow powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.94 (1H, s, 2-H), 7.64 (1H, s, 5-H), 7.37 (2H, d, J = 8.5 Hz, 2′,6′-H), 6.89 (1H, s, 8-H), 6.84 (2H, d, J = 8.5 Hz, 3′,5′-H), 4.00, 3.99 (3H each, both s, $OCH_3 \times 2$). FAB-MS (m/z): 299 $[M+H]^+$. HR FAB-MS (m/z): calcd for $C_{17}H_{14}O_5$+H: 299.2901, found: 299.2905.
12. In 6-well microculture plates, tectorigenin (**1**) was co-incubated with HMC-1 cells (5.0×10^5 cells/mL) in 3 mL of the IMDM medium used for the bioassay at the concentration of 50 μM at 37 °C in 5% CO_2 for 36 h. Total cellular RNA was isolated using QuickPrep micro mRNA Purification Kit (Amersham) according to the manufacturer's instructions. First strand cDNA was synthesized using anchored oligo(dT) primers and ReverTra Ace reverse transcriptase (TOYOBO). The resultant cDNAs were respectively amplified by using the following primers: FcεRIα forward, 5′ataaaagctccgcgtgagaa3′, reverse, 5′tccttgagcacagacgtttc3′; FcεRIβ forward, 5′ttaccaggacctctaggagtgg3′, reverse 5′aggctggatgaaaaggtgtt3′; FcεRIγ forward 5′ccagcagtggtcttgctcttact3′, reverse 5′gcatgcaggcatatgtgat gcca3′; G3PDH forward 5′gatgacatcaagaaggtggtg3′, reverse 5′gctgtagccaaa ttcgttgtc3′. The amplified PCR products were subjected to electrophoresis on a 1.5% TAE (Tris-acetate EDTA buffer) agarose gel, then stained with ethidium bromide. Image analysis for each blot was conducted by Scion image (Scion) to determine amount of the expressed mRNA and quantification of each mRNA was carried out in triplicate. G3PDH was used as a control to correct expression of FcεRIα, β, and γ. The relative expression rates of the three subunits were described as compared with that of unstimulated HMC-1 cells.