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Original Article

Differentiation between control subjects and patients with chronic spontaneous urticaria based on the ability of anti-IgE autoantibodies (AAbs) to induce FcεRI crosslinking, as compared to anti-FcεRIα AAbs

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AAbs, autoantibodies; ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; EXiLE test, IgE crosslinking-induced luciferase expression test; h, human; FcεRIα, FcεRI α-chain; NC, non-atopic healthy control; NF-AT, nuclear factor of activated T-cell; PBST, PBS containing 0.05% Tween 20; PMA, phorbol 12-myristate 13-acetate; r, recombinant; ROC, receiver operator characteristic; soluble FcεRIα, soluble form of FcεRI α-chain ectodomain; SCF, stem cell factor

ABSTRACT

Background: The reported prevalences of IgG autoantibodies (AAbs) to FcεRIα and IgE in sera from patients with chronic spontaneous urticaria (CSU) have varied, and these AAbs are also often observed in healthy control subjects. Regarding the histamine release activity of purified IgG from patients with CSU, the number of examined patients has been small. Thus, we sought to determine the prevalence and FcεRI crosslinking ability of these AAbs in a large number of patients with CSU and non-atopic control (NC) subjects.

Methods: We compared the concentrations of anti-IgE and anti-FcεRIα AAbs and the abilities of these AAbs to cause FcεRI aggregation in patients with CSU (n = 134) and NC subjects (n = 55) using ELISA and an *in vitro* elicitation test, respectively.

Results: The concentration of anti-IgE AAbs was significantly different between the NC subjects and the CSU patients ($P < 0.0001$, cutoff value: 0.558 μg/mL), whereas the concentration of anti-FcεRIα AAbs was not. A significant difference in the duration of illness was noted between patients with lower and those with higher concentrations of anti-IgE AAbs relative to the cutoff value. The abilities of anti-IgE AAbs, but not anti-FcεRIα AAbs, to induce FcεRI crosslinking were significantly higher in CSU patients than in NC subjects ($P = 0.0106$).

Conclusions: In the Japanese population of CSU patients studied, the ability of the anti-IgE AAbs to induce FcεRI crosslinking differed significantly between NC subjects and CSU patients, suggesting the involvement of anti-IgE AAbs in the pathogenesis of CSU in the Japanese population.

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Introduction

Chronic spontaneous urticaria (CSU) is defined as the occurrence of systemic daily wheals for at least 6 weeks.[1] Since the presence of IgG autoantibodies (AAbs) to FcεRI α-chain (FcεRIα) and IgE has been repeatedly observed in patients with CSU,[1,2] autoimmunity is thought to be one of the major causes of CSU.[3] However, the prevalence of anti-FcεRIα and anti-IgE AAbs in sera ranged from 4% to 64% and from 0% to 69% among CSU patients and from 0% to 43% and from 0% to 30% among healthy controls, respectively[4] and these AAbs have also been observed in not only healthy control subjects but also patients with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.[5,6] Recently, Kolkhir *et al*. assessed existing evidence supporting the role for autoimmunity in CSU using Hill's criteria of causality for level 1 (causal relationship) to level 5 (causality not likely).[4] The IgG AAbs type of autoimmunity in patients with CSU has been assessed to have a level 2 causality (causal relationship likely).

Several studies have demonstrated that sera from some patients with CSU induce histamine release from human basophils and human mast cell lines.[7–11] However, sera contain complement and other factors that may induce histamine release and augment IgG-dependent histamine release from basophils.[7,12] Regarding the histamine release activity of purified IgG from patients with CSU, the number of examined patients has been small. Purified IgG from patients with CSU induced histamine release in four out of eight patients[13] and in six out of 11 patients.[7] Therefore, to evaluate the ability of anti-FcεRIα or anti-IgE AAbs to induce the crosslinking of FcεRI on mast cells and basophils, purified IgG from a relatively large number of patients with CSU and healthy control subjects is needed. Furthermore, the IgE-dependent reactivity of basophils depends on the donors of the basophils. Which anti-FcεRIα or anti-IgE AAbs activate basophils to induce histamine release is not distinguishable using basophils unless IgE stripping has been performed. Thus, a recently developed luciferase-reporting mast cell line (RS-ATL8), which express human FcεRI, to detect IgE crosslinking-induced luciferase expression (EXiLE),[14] was used and modified to measure mast cell activation by anti-FcεRIα and anti-IgE AAbs from patients with CSU (designated as a modified EXiLE test).

Methods

Ethical considerations

This study was approved by the Ethics Committees of the Nihon University School of Medicine (RK-15908-12 and RK-160112-2). All the subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Patient enrollment

One hundred and thirty eight patients with CSU (93 females and 45 males; ranging in age from 13 to 87 years) were consecutively recruited between October 2009 and August 2017. Four patients were excluded because steroids had been used within 3 weeks. The characteristics of one hundred and thirty four patients with CSU (90 females and 44 males; ranging in age from 13 to 87 years) are summarized in Table 1. The majority of patients were referred from private practice doctors to the outpatient clinic of Nihon University Hospital. Some patients had already received treatment with H1-antihistamines. We also enrolled 55 non-atopic healthy control (NC) subjects (32 females and 23 males; ranging in age from 22 to 57 years) whose criteria were no history of urticaria, asthma, allergic diseases, or autoimmune diseases (Table 1). Routine blood and urine tests and liver and kidney function were all normal. Women who were pregnant or nursing were excluded.

Table 1
Characteristics of the patients with CSU and NC subjects.

Characteristics	CSU (n = 134)	NC (n = 55)
Demographic details		
Age (y), median (range)	44 (13–87)	31 (22–57)
Sex: female (%)	67.2	58.2
Urticaria		
Durations of illness (Mo), median (range)	26.5 (2–480)	NA
Severity score, median (range)	3 (3–6)	NA
Medication use at time of review (%)		
H1 antihistamine	94.0	0
H2 antihistamine	35.8	0
Leukotriene receptor antagonist (montelukast)	19.4	0
Corticosteroid	0	0
Cyclosporine A	0	0
Tranexamic acid	0	0
Dapsone	0.75	0
Nil	1.49	100

NA, Not applicable.

Definitions and study protocol

The diagnosis of CSU was made according to the classification of the European Academy of Allergy and Clinical Immunology (EAACI), the Global Allergy and Asthma European Network (GA[2] LEN), the European Dermatology Forum (EDF), and the World Allergy Organization (WAO).[15] Subjects were excluded if steroids or immunosuppressive drugs had been used within 3 weeks. The UAS7 is a patient-reported scoring system that captures the severity of pruritus and the number of hives appearing during 1 week.[16] The intensity of pruritus (range, 0 [none] to 3 [severe]) and the number of hives (range, 0 [none] to 3 [>50 hives]) were recorded daily (maximum, 6 points per day). The scores were then summed for 1 week to represent the UAS7 scale, (0–42). To estimate urticarial activity, urticaria severity scores were also used according to the guidelines of the Japanese Dermatology Association (Supplementary Table 1).[17] Blood tests, which included serum IgE levels, basophil counts, IgG anti-nuclear AAbs, IgG anti-thyroglobulin AAbs, and IgG anti-thyroid microsomal AAbs, were performed.

Autologous serum skin test (ASST)

The ASST was performed using 50 μL of the patient's own serum intradermally injected into the flexor aspect of the forearm; 50 μL of saline was injected 3–5 cm away as a control. The results were measured after 30 min. If the serum-injected site manifested a wheal with a diameter of 1.5 mm greater than that of the saline-injected site, the result was considered positive.[18] According to the EAACI/GA (2)LEN task force consensus report,[18] antihistamines were discontinued for 2–3 days prior to the ASST. The sera used for the ASST were also used for measurement of concentrations of the AAbs and the abilities of the AAbs to induce FcεRI-crosslinking

Isolation of IgG fraction from serum

IgG was purified from sera of patients with CSU and NC subjects using Ab-Rapid SPiN EX (ProteNova, Kagawa, Japan) according to the manufacturer's protocol. The purified IgG was reconstituted with PBS using Amicon centrifugal filter units (Merck Millipore, Darmstadt, HE, Germany). The final concentrations of the purified IgG were diluted to one-sixth of the serum IgG concentrations.

Expression of recombinant human (rh) soluble form of FcεRIα ectodomain (soluble FcεRIα) and purification of secreted rh soluble FcεRIα

The truncated human FcεRIα ectodomain (1–172 amino acids) was secreted from transfected Chinese hamster ovary (CHO) cells as described.[19] Secreted rh soluble FcεRIα was purified from the culture supernatant of the cells using mouse anti-FcεRIα (clone CRA2).[20] rh soluble FcεRIα was eluted using an acid buffer (0.1 M glycine/0.2 M NaCl, pH 2.7), immediately neutralized using a basic buffer (1 M Tris–HCl, pH 9), quantified using Bradford method, and applied to a gradient gel for SDS-PAGE, as described previously.[19]

Generation of humanized anti-human FcεRIα mAb, clone CRA2

The anti-human FcεRIα mAb, clone CRA2,[20] was humanized by complementarity-determining region grafting onto human V region frameworks encoded by human germline V and J genes, as described previously.[21] Appropriate light and heavy chain expression vectors were co-transfected into COS7 cells using Lipofectamine reagent (Life Technologies, Grand Island, NY), as described previously.[21] The purification of recombinant mAb was performed as described.[20]

Measurement of concentrations of anti-FcεRIα AAbs

An ELISA for detecting anti-human FcεRIα AAbs was performed as previously described[6,22–24] with the exception that a standard curve for IgG anti-FcεRIα was generated using humanized anti-FcεRIα mAb (clone CRA2, isotype human IgG1).[21] Briefly, 1 μg/mL of purified recombinant soluble FcεRIα proteins[19] in sodium carbonate, pH 9.6 (Calbiochem, San Diego, CA), were used to coat polystyrene microwells of an ELISA plate (Maxisorp; Nunc, Roskilde, Denmark) in duplicates and were incubated overnight at 4 °C. The plate was washed 4 times with PBS containing 0.05% Tween 20 (PBST) and then blocked with 10% FBS in PBS for 1 h at room temperature. After washing, the purified IgG from the sera of NC subjects and patients with CSU were added at a 1:10 dilution with PBS to each well, then incubated for 2 h at room temperature. After washing, HRP-conjugated anti-human IgG mAb (clone G18-145; BD Pharmingen, Franklin, NJ), HRP-conjugated anti-human IgG1 mAb (clone HP6069; ThermoFisher Scientific, Waltham, MA), or HRP-conjugated anti-human IgG4 mAb (clone HP6025; ThermoFisher Scientific) was added to each well at a 1:10,000 dilution for measuring its concentration of total IgG, IgG1 or IgG4, respectively, then incubated for 1 h at room temperature. The reactions in the wells were developed with hydrogen peroxide plus 3,3′,5,5′-tetramethylbenzidine in 100 mM citrate-phosphate buffer, pH 5.0 (KPL, Gaithersburg, MD), and their absorbances were monitored at 450 nm and 570 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific) after the addition of 2 N sulfuric acid. The linear region of the standard curve was used to quantify the anti-FcεRIα in the purified IgG.

Measurement of concentrations of anti-IgE AAbs

An ELISA was used to detect anti-IgE AAbs, as previously described.[5,25–27] Briefly, the polystyrene microwells of an ELISA plate (Maxisorp) were coated with human myeloma IgE (1 μg/mL in sodium carbonate pH 9.6, Calbiochem) and incubated overnight at 4 °C. Unbound protein was removed by four washes with PBST. Free binding sites were blocked by incubation with 10% FBS in PBS for 1 h at room temperature. After washing, the purified IgG from the sera of NC subjects and patients with CSU were added at a 1:10 dilution with PBS to each well, which were then incubated for 2 h at room temperature. After washing, HRP-conjugated anti-human IgG mAb (clone G18-145) was added to each well at a 1:10,000 dilution, which was then incubated for 1 h at room temperature. The reactions in the wells were developed as mentioned above. The quantity of reactive IgG was calculated using standard amounts of purified human IgG (Jackson Immuno Research Laboratories, West Grove, PA) ranging from 0.775 ng/mL to 50 ng/mL bound directly to the plates. To normalize the data among the plates, one specific donor (CSU donor# 35)'s anti-IgE AAbs level was measured repeatedly on all of the plates.

Preparation of RS-ATL8 cells[28] and RBL-NL4 cells

RS-ATL8 cells or RBL-NL4 cells (2.0×10^5 cells/1000 μL/well) were cultured in MEM containing 10% FBS, 1% GlutaMAX-I, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 mg/mL geneticin and 0.2 mg/mL hygromycin B on a Costar® 24-well clear tissue culture-treated multiple well plate (Corning Inc., Corning, NY) at 37 °C for 2 days in a 5% CO_2 incubator.[28] The cells were re-plated and cultured as described above for an additional 2 days. The cells were then re-plated on a 96-well plate at 2×10^4 cells/50 μL/well and cultured at 37 °C overnight in a 5% CO_2 incubator.

Measurement of the ability of anti-FcεRIα and anti-IgE AAbs to induce FcεRI crosslinking using an in vitro elicitation test (modified EXiLE test)

An *in vitro* elicitation test was modified from a previously described method reported by us.[28] The test was performed by assessing an EXiLE test using human FcεRIα$\beta\gamma_2$ genes- and nuclear factor of activated T-cell (NF-AT)-responsive luciferase reporter gene-introduced rat basophilic leukemia cells (RS-ATL8 cells).[14] The purified IgG was replaced with MEM containing 10% FBS, 1% GlutaMAX-I, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 mg/mL geneticin and 0.2 mg/mL hygromycin B, using Amicon centrifugal filter units (Merck Millipore). To measure the ability of anti-FcεRI AAbs to induce FcεRI crosslinking, 50 μL of purified IgG (equivalent to a 1:6 dilution of the serum IgG levels) obtained from NC subjects or CSU patients was added to each well and the samples were incubated at 37 °C for 3 h in a 5% CO_2 incubator. To measure the ability of anti-IgE AAbs to induce FcεRI-crosslinking, the purified IgG was pre-incubated with well-coated rh soluble FcεRIα (1 μg/mL), and the supernatants were sequentially incubated with well-coated rh soluble FcεRIα twice. Then, the supernatants were applied to IgE-sensitized RS-ATL8 cells. Fifty μL of luciferase substrate solution containing a cell lysis reagent (ONE Glo, Promega, Madison, WI) was added to the cells, and the chemiluminescence was measured using a Centro LB 960 plate reader (Berthold Technologies, Bad Wildbad, Germany). Standard curves for the fold inductions in luciferase fluorescence were induced by mouse anti-human FcεRIα mAb (clone CRA1; eBioscience, San Diego, CA) or rabbit anti-human IgE Ab (Dako, Santa Clora, CA). The luciferase expression levels were represented as the fold increase in light units compared with the results in non-stimulated cells. The minimum concentration of CRA1 or rabbit anti-human IgE Ab that induced a significantly higher intensity of luciferase fluorescence than that observed in the non-stimulated wells was used to define as the sensitivity of the EXiLE test.

Removal of anti-FcεRIα AAbs from purified IgG obtained from CSU patients

To examine whether anti-FcεRIα AAbs were directly bound to cell surface FcεRI on RS-ATL8 cells, purified IgG from the sera of patients with CSU was pre-incubated with well-coated rh soluble

FcεRIα (1 μg/mL), and the supernatants were sequentially incubated with well-coated rh soluble FcεRIα twice. As positive control, humanized anti-FcεRIα mAb (clone CRA2) in place of the anti-FcεRIα AAbs obtained from patients with CSU was similarly pre-incubated in wells coated with rh soluble FcεRIα. Then, the supernatants were applied to the RS-ATL8 cells for use in the modified EXiLE test.

Removal of anti-IgE AAbs from purified IgG obtained from CSU patients

To examine whether anti-IgE AAbs bind directly to cell surface-bound IgE on RS-ATL8 cells, anti-FcεRIα AAbs-absorbed purified IgG from sera obtained from patients with CSU was pre-incubated with well-coated human myeloma IgE (1 μg/mL, Calbiochem), and the supernatants were sequentially incubated with well-coated human myeloma IgE twice. Then, the supernatants were applied to IgE-sensitized RS-ATL8 cells for the modified EXiLE test.

Basophil isolation, purification and basophil activation test

Blood samples were collected from 3 healthy donors. Basophil-enriched suspensions were obtained by Percoll (GE Healthcare UK, Buckinghamshire, England) density gradient centrifugation. Briefly, basophil-enriched preparations were isolated from whole fresh blood. Blood was layered over a two-step discontinuous Percoll gradient consisting of 15 mL of 62% Percoll overlaid with 15 mL of 53% Percoll prepared in 50 mL tubes and centrifuged. A basophil rich layer, which was located 1 cm above the 53/62% interface, was harvested.[29] Furthermore, basophil enrichment was carried out by using a negative selection method with the Human Basophil Isolation Kit II (Milteny Biotec, Bergisch Gladbach, Germany).[30] The purified basophils (purity; 70–94%) were suspended in Hepes buffer. The number of basophils used for the experiments was adjusted to 2×10^4 cells/100 μL. The purified basophils were stimulated with CRA1, rabbit anti-human IgE Ab (Dako) or N-formyl-methionyl-leucyl-phenylalanine (fmlp; Sigma-Aldrich, St. Louis, MO) for 30 min at 37 °C. Activation was stopped by chilling on ice for 5 min. Thereafter, the cells were labeled with FITC-conjugated anti-CD123 (Biolegend, San Diego, CA) and PE-conjugated anti-CD203c (Milteny Biotec) and incubated for 20 min on ice. Finally, cells were washed and analyzed on a flow cytometer. Only basophils with bright CD123 expression were gated. Data on at least 1000 basophils were acquired, and the percentage of CD203c-expressing basophils was calculated.[31]

Statistical analysis

To identify variables associated with CSU or a specific CSU patient group, we compared continuous variables between the NC subjects and patients with CSU or between two different patient groups using the Mann-Whitney-*U* test; to compare categorical variables, a 2-sided Fisher exact test was used. We estimated the effects on the odds ratio using a 2-sided Fisher exact test. The receiver operator characteristic curve (ROC curve) was used to determine the optimal cutoff values that maximized the sum of the specificity and sensitivity. Spearman rank correlation coefficients were calculated to determine the strength of the correlations between continuous variables. *P* value was considered significant at $P < 0.05$. The data analyses were performed using Prism version 7 (GraphPad software; San Diego, CA).

See the Supplementary Methods for more information.

Results

Comparison of concentrations of anti-FcεRIα and anti-IgE AAbs between NC subjects and patients with CSU

We first compared the concentrations of anti-FcεRIα and anti-IgE AAbs between 55 NC subjects and 134 patients with CSU. The concentrations of anti-IgE AAbs were significantly different between the NC subjects and the CSU patients (Fig. 1B, C; $P < 0.0001$; odds ratio, 7.14 with a cutoff value of 0.558 μg/mL, sensitivity, 76.2%; specificity, 69.1% by ROC curve analysis comparing NC subjects and CSU patients), but those of anti-FcεRIα AAbs were not (Fig. 1A). A significant correlation was not seen between the

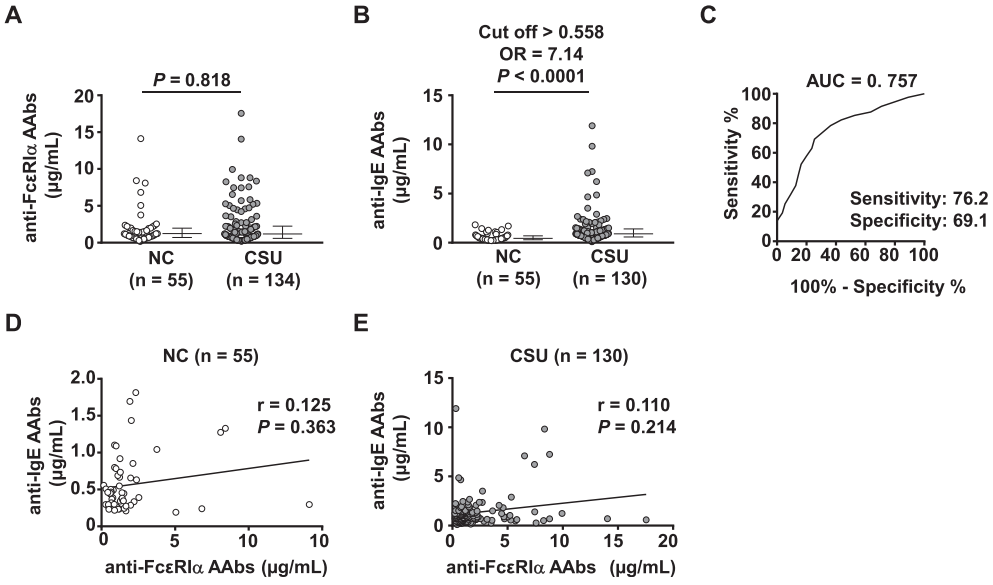

Fig. 1. Comparison of concentrations of anti-FcεRIα **(A)** and anti-IgE AAbs **(B)** between NC subjects and patients with CSU (scatter plot). **(C)** ROC curve analyses for stratifying CSU patients according to the concentration of anti-FcεRIα and anti-IgE AAbs; at a calculated cutoff of 0.558 μg/mL, the assay provided the best discrimination between NC subjects and CSU patients ($P < 0.0001$) and the optimal values for sensitivity (76.2%) and specificity (69.1%). The medians and inter-quartile ranges are also shown. OR, odds ratios. Correlation between concentrations of anti-IgE to anti-FcεRIα AAbs in NC subjects **(D)** and CSU patients **(E)**.

concentrations of anti-FcεRIα and anti-IgE AAbs in both NC subjects (Fig. 1D) and patients with CSU (Fig. 1E).

Next, to clarify the clinical characteristics between CSU patients with lower and higher concentrations of anti-IgE AAbs relative to the cutoff value, we compared the variables between these two groups of CSU patients. While no significant differences in the patient age, urticaria severity scores, frequency of positive results of ASST, or clinical data including the serum IgE levels, basophil counts, the concentrations of anti-FcεRIα AAbs, and positive rates of IgG AAbs to nucleus, thyroglobulin, and thyroid microsomal protein were observed between the two patient groups, a significant difference in the duration of illness was noted between the two groups, as shown in Table 2 ($P = 0.0101$).

Evaluation of ability of anti-FcεRIα to induce FcεRI aggregation in patients with CSU, compared with NC subjects

Next, we evaluated the ability of anti-FcεRIα to induce FcεRI aggregation in NC subjects and patients with CSU using the modified EXiLE test. We evaluated the accuracy of this assay. Figure 2A shows the standard curve for the fold inductions in luciferase fluorescence induced by mouse anti-human FcεRIα mAb (clone CRA1). As a positive control, the fold inductions in luciferase fluorescence induced by phorbol 12-myristate 13-acetate (PMA) + ionomycin were used (Supplementary Fig. 1A). To clarify that the effect of anti-FcεRIα AAbs was mediated by cell surface FcεRI on RS-ATL8 cells, purified IgG from patients with CSU was preincubated with well-coated rh soluble FcεRIα, and the resulting supernatants were used in the modified EXiLE test. The results clearly showed that removal of anti-FcεRIα AAbs from purified IgG obtained from the CSU patients significantly blocked the effects of FcεRI crosslinking induced by anti-FcεRIα AAbs (Fig. 2B-a, $P = 0.0039$). Pre-incubation of humanized anti-FcεRIα mAb (clone CRA2) with soluble FcεRIα completely blocked the effects (Fig. 2B-b). Also, we confirmed that humanized anti-FcεRIα mAb (clone CRA2) induced less than 1.5-fold increase of luciferase fluorescence in the control cells, which consisted of NF-AT-responsive luciferase reporter gene-introduced rat basophilic leukemia cells without the induction of human FcεRIαβγ$_2$ genes (RBL-NL4 cells), and the fold increases in the luciferase fluorescence intensity were not dependent on the concentration of CRA2 (Supplementary Fig. 1B). PMA + ionomycin induced ~350-fold increase of luciferase fluorescence (Supplementary Fig. 1C). We used purified IgG (equivalent to a 1:6 dilution of the serum IgG levels) from NC subjects or CSU patients in the assays to compare the ability of the anti-FcεRIα AAbs to induce FcεRI aggregation and to avoid the effects of other components, such as complement and IgE to autoantigens in the sera.[32,33] As can be seen in Figure 2C, anti-FcεRIα AAbs from the CSU patients did not induce significantly more FcεRI crosslinking than anti-FcεRIα AAbs from the NC subjects ($P = 0.131$). Only 4 of the 134 patients with CSU exhibited higher values than the highest value observed in the NC subjects (2.117-fold increase in the ability of anti-FcεRIα AAbs to induce FcεRI crosslinking). The maximum effects of the anti-FcεRIα AAbs from patients with CSU were almost equivalent to those induced by ~10 ng/mL of CRA1 (Fig. 2A). Significant correlations were not seen between the concentrations of anti-FcεRIα AAbs and the levels of FcεRI crosslinking in NC subjects and CSU patients (Fig. 2D, E). There were no significant differences in clinical parameters between CSU patients with over 2.117 fold increase of the ability of anti-FcεRIα AAbs to induce FcεRI crosslinking and patients with lower 2.117 fold increase of the ability of it (data not shown).

Table 2
Comparison of patient characteristics between CSU patients with lower and higher concentrations relative to the cutoff value for anti-IgE AAbs.

	anti-IgE AAbs (μg/mL)		*P* value
	<0.558	≧ 0.558	
Number	31	99	
Female sex (%)	77.4	66.7	0.373‡
Age (years), median (range)	42 (19–76)	44 (13–87)	0.729§
Durations of illness (months), median (range)	12 (2–240)	30 (2–480)	0.0101†,§
ASST positive (%)	40	40.7	>0.999‡
Severity scores, median (range)	3.5 (3–5)	3 (3–6)	0.186§
UAS7, median (range)	28 (28–28)	28 (0–42)	>0.999§
Serum IgE level (IU/mL, mean ± SD)	226.4 ± 213.7	406.6 ± 627.4	0.173§
Blood basophils (mm^3, mean ± SD)	28.5 ± 16.8	36.2 ± 33.1	0.411§
Anti-nuclear AAbs positive (%)	16.7	14.3	0.750‡
Anti-thyroglobulin AAbs positive (%)	13.0	7.9	0.430‡
Anti-thyroid microsomal AAbs positive (%)	22.7	10.2	0.266‡
Anti-FcεRIα AAbs (μg/mL, mean ± SD)	1.71 ± 2.03	2.36 ± 2.93	0.199§

SD, standard deviation.
† Statistical significance between CSU patients with a value lower than the cutoff value for anti-IgE AAbs concentrations and patients with a value higher than the cutoff value.
‡ Fisher exact test.
§ Mann Whitney U test.

Evaluation of ability of anti-IgE AAbs to induce FcεRI aggregation in patients with CSU, compared with NC subjects

Next, we similarly evaluated the ability of anti-IgE AAbs to induce FcεRI aggregation in NC subjects and patients with CSU using the modified EXiLE test. We evaluated the accuracy of this assay. Figure 3A shows the standard curve for the fold inductions in luciferase fluorescence induced by rabbit anti-human IgE Ab. To clarify that the effect of anti-IgE AAbs was mediated by cell surface-bound IgE on RS-ATL8 cells, purified IgG from patients with CSU was preincubated with well-coated human myeloma IgE and soluble FcεRIα, and the resulting supernatants were used in the modified EXiLE test. The results clearly showed that the removal of both anti-IgE and anti-FcεRIα AAbs from purified IgG obtained from CSU patients significantly blocked the effects of FcεRI crosslinking induced by anti-IgE AAbs (Fig. 3B-a; $P = 0.0039$). Pre-incubation of rabbit anti-human IgE Ab with human myeloma IgE and soluble FcεRIα blocked the effects (Fig. 3B-b). We used purified IgG (equivalent to a 1:6 dilution of the serum IgG levels) from NC subjects or CSU patients in the assays. To avoid the effects of anti-FcεRIα AAbs, anti-FcεRIα AAbs in purified IgG were absorbed. For IgE sensitization, the RS-ATL8 cells were incubated with human myeloma IgE for 30 min at 37 °C. As can be seen in Figure 3C, anti-IgE AAbs from the CSU patients induced significantly more FcεRI crosslinking than anti-IgE AAbs from the NC subjects ($P = 0.0106$; odds ratio, 2.51) with a cutoff value of a 1.302-fold increase derived from a ROC analysis comparing NC subjects and CSU patients (Fig. 3D; sensitivity, 70.8% and specificity, 50.9%). Seventy percent of the patients with CSU exhibited a value that was over the cutoff value for the discrimination of the ability anti-IgE AAbs to induce FcεRI crosslinking. The maximum effects of the anti-IgE AAbs from patients with CSU were almost equivalent to those induced by 0.3 μg/mL of rabbit anti-human IgE Ab (Fig. 3A). Significant correlations were not seen between the concentrations of anti-IgE AAbs and the levels of FcεRI crosslinking in NC subjects and CSU patients (Fig. 3E, F). There were no significant differences in clinical parameters between CSU patients with a value lower than the cutoff value for the discrimination of the ability of anti-IgE AAbs to induce FcεRI crosslinking (<1.302-fold) and patients with a value higher than the cutoff value (≥1.302-fold, Table 3). Although no significant correlations were observed between the ability of anti-FcεRIα and anti-IgE AAbs to induce FcεRI aggregation in NC subjects (Fig. 3G)

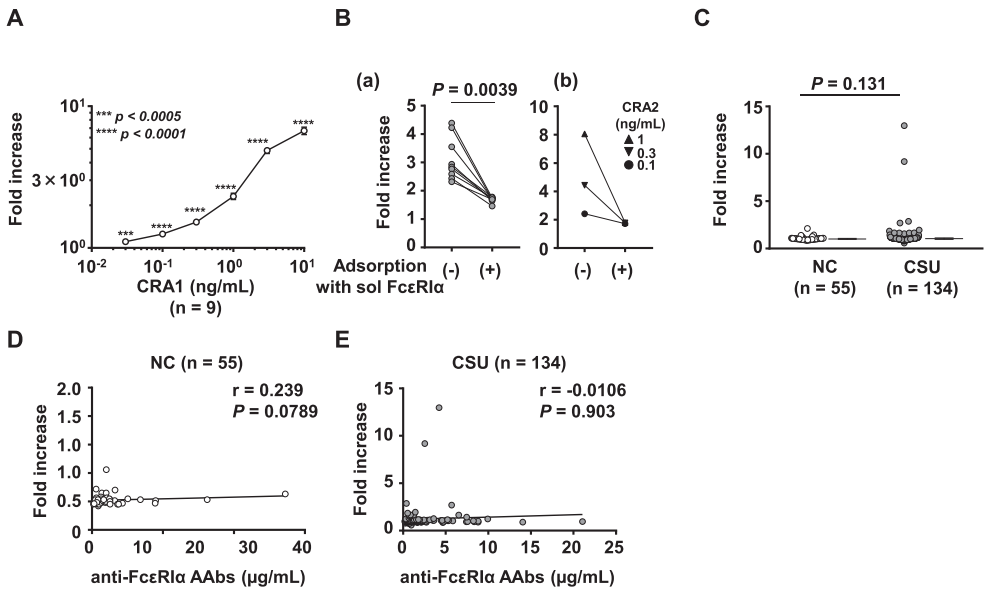

Fig. 2. Evaluation of ability of anti-FcεRIα AAbs to induce FcεRI aggregation in patients with CSU, compared with NC subjects. **(A)** Standard curve for the fold inductions in luciferase fluorescence induced by mouse anti-human FcεRIα mAb (clone CRA1). The results are shown as the mean ± SEM of 9 independent experiments. **(B)** The fold inductions of luciferase chemiluminescence by anti-FcεRIα AAbs-stimulation were dependent on cell surface FcεRI on mast cells. The purified IgG from patients with CSU **(a)** or CRA2 **(b)** was preincubated with well-coated rh soluble FcεRIα, and the resulting supernatants were used in the modified EXiLE test. sol FcεRIα, soluble FcεRIα. **(C)** Comparison of ability of anti-FcεRIα AAbs to induce FcεRI aggregation between NC subjects and patients with CSU (scatter plot). The medians and inter-quartile ranges are also shown. **(D)** and **(E)** Correlation between the concentrations of anti-FcεRIα AAbs and the fold increases in NC subjects **(D)** and CSU patients **(E)**.

and CSU patients (Fig. 3H), the ability of anti-IgE AAbs to induce FcεRI aggregation tended to be correlated with the ability of anti-FcεRIα AAbs to induce FcεRI aggregation.

Comparison of the modified EXiLE test with the histamine release assay in human basophils and cultured synovium-derived mast cells following FcεRI aggregation

The values of the modified EXiLE test were compared with the results of the histamine release assay for human basophils and human synovium-derived cultured mast cells in response to 0.03–1000 ng/mL of mouse anti-human FcεRIα mAb (clone CRA1) or 0.01–10 μg/mL of rabbit anti-human IgE Ab. Human basophils were activated in response to 3–1000 ng/mL of CRA1 (Fig. 4A). Comparison of the results of the basophil activation test (Fig. 4A) and modified EXiLE test for CRA1 (Fig. 2A) revealed a 30- to-100-fold higher sensitivity of the latter for detecting FcεRI cross-linking. The human synovium-derived cultured mast cells induced histamine release in response to 3–1000 ng/mL of CRA1 (Fig. 4C). Human basophils and human synovium-derived cultured mast cells were activated in response to 0.03–10.00 μg/mL and 0.01–10.00 μg/mL of anti-human IgE Ab, respectively (Fig. 4B, D), indicating that the anti-IgE AAbs from patients with CSU had the ability to induce histamine release from both human basophils and synovium-cultured mast cells.

Discussion

This is the first definite report to demonstrate that the abilities of anti-IgE AAbs in purified IgG obtained from a large number of patients with CSU to induce FcεRI crosslinking were significantly higher than those of anti-IgE AAbs from NC subjects (Fig. 3C); however, similar results were not observed for anti-FcεRIα AAbs (Fig. 2C). When the modified EXiLE tests was compared to activation assays for human basophils and synovium-derived cultured mast cells (Fig. 4), the modified EXiLE test was found to be appropriate for evaluating the crosslinking of FcεRI by anti-IgE AAbs in a disease context.

Our observation suggests that there is something qualitatively different about these AAbs in the sera of patients with CSU that causes them to crosslink FcεRI and thus could be a mechanism of the CSU for this population of patients with CSU. The finding that there was no correlation between the concentrations of anti-IgE AAbs and the ability of the AAbs to induce FcεRI crosslinking in purified IgG from patients with CSU (Fig. 3E) suggests that only a small fraction of the total AAbs are responsible for driving the disease *per se*. Anti-FcεRIα and anti-IgE AAbs from patients with CSU would contain polyclonal Abs in nature and might contain a mixture of anti-FcεRIα and anti-IgE AAbs with different biological functions, including different isotypes. Thus, these findings support the hypothesis that either the avidity of the offending AAbs is different from that of the innocuous ones, that the bound epitopes differ, or that the proportion of isotypes of the AAbs differ. We found that the avidity of anti-FcεRIα AAbs from patients with CSU was significantly higher than that from NC subjects (Supplementary Fig. 2A, $P = 0.012$), but there was no significant correlation between the avidity of anti-FcεRIα AAbs and the ability of anti-FcεRIα AAbs to induce FcεRI crosslinking in patients with CSU (Supplementary Fig. 2B). Also, the ratio of IgG1 anti-FcεRIα AAbs to IgG4 anti-FcεRIα AAbs between NC subjects and patients with CSU were not significant (Supplementary Fig. 2C). Although we were unable to investigate the difference in the epitopes of these AAbs between NC subjects and patients with CSU, the different abilities of anti-FcεRIα and anti-IgE AAbs to induce FcεRI crosslinking may be due to the mixtures of different avidities, epitopes and isotypes of the offending and innocuous AAbs in patients with CSU. Further studies are needed to clarify the qualitative difference in anti-FcεRIα and anti-IgE AAbs between NC subjects and patients with CSU.

Since complement was excluded in our modified EXiLE test for measuring the ability of these AAbs to induce FcεRI crosslinking by using purified IgG, the fold increases were induced by IgG alone. Another factor that would affect the modified EXiLE test is cross-linking between FcγRIIb and FcεRI. Human IgGs reportedly bind to

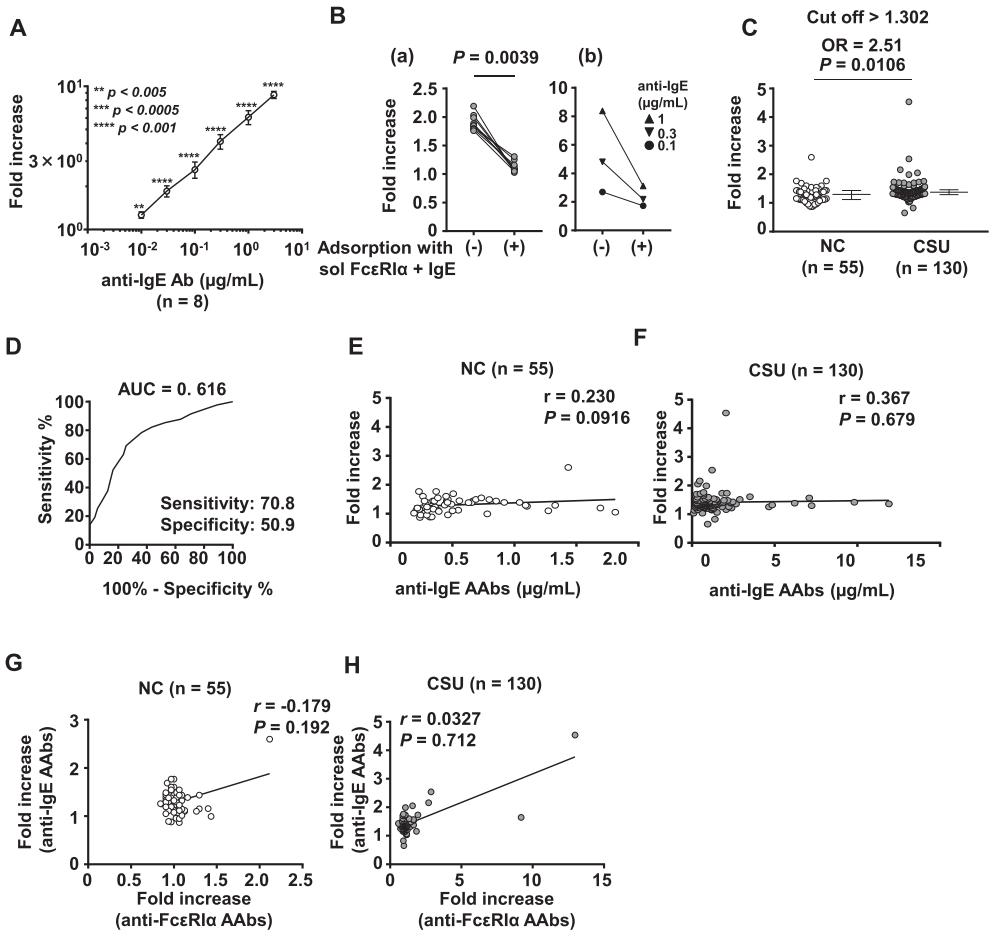

Fig. 3. Evaluation of ability of anti-IgE AAbs to induce FcεRI aggregation in patients with CSU, compared with NC subjects. **(A)** Standard curve for the fold inductions in luciferase fluorescence induced by rabbit anti-human IgE Ab. The results are shown as the mean ± SEM of 8 independent experiments. **(B)** Anti-FcεRIα AAbs in purified IgG were absorbed. The purified IgG from patients with CSU **(a)** or rabbit anti-human IgE polyclonal Ab **(b)** was preincubated with well-coated human myeloma IgE, and the resulting supernatants were used in the modified EXiLE test. sol FcεRIα, soluble FcεRIα. **(C)** Comparison of ability of anti-IgE AAbs to induce FcεRI aggregation between NC subjects and patients with CSU (scatter plot). The medians and inter-quartile ranges are also shown. **(D)** ROC curve analysis for stratifying CSU patients according to the fold increases (modified EXiLE test); at the calculated cutoff of a 1.302-fold increase, the assay provided the best discrimination between NC subjects and CSU patients ($P = 0.0106$) and the optimal values for sensitivity (70.8%) and specificity (50.9%). **(E)** and **(F)** Correlation between the concentrations of anti-IgE AAbs to the fold increases in NC subjects **(E)** and CSU patients **(F)**. **(G)** and **(H)** Correlation between the ability of anti-FcεRIα and anti-IgE AAbs to induce FcεRI aggregation in NC subjects **(G)** and CSU patients **(H)**.

Table 3
Comparison of patient characteristics between CSU patients with a value lower than the cutoff value for the discrimination of the ability of anti-IgE AAbs to induce FcεRI crosslinking and patients with a value higher than the cutoff value.

	Fold increase		*P* value
	<1.302	≧ 1.302	
Number	38	92	
Female sex (%)	79.0	65.2	0.147†
Age (years), median (range)	45 (13–76)	42 (19–87)	0.482‡
Durations of illness (months), median (range)	31 (2–480)	24 (2–300)	0.783‡
ASST positive (%)	33.3	43.6	0.461†
Severity scores, median (range)	3 (3–6)	3 (3–6)	0.199‡
UAS7, median (range)	20 (8–42)	29 (0–42)	0.444‡
Serum IgE level (IU/mL, mean ± SD)	241.8 ± 230.8	421.7 ± 656.4	0.140‡
Blood basophils (mm^3, mean ± SD)	40.8 ± 45	31.9 ± 21.4	0.634‡
Anti-nuclear AAbs positive (%)	16.1	14.1	0.769†
Anti-thyroglobulin AAbs positive (%)	10.3	8.6	0.719†
Anti-thyroid microsomal AAbs positive (%)	4.2	19.6	0.148†
Anti-FcεRIα AAbs (μg/mL, mean ± SD)	1.94 ± 2.30	2.31 ± 2.91	0.253‡
Anti-IgE AAbs (μg/mL, mean ± SD)	1.21 ± 1.29	1.40 ± 1.81	0.830‡

SD, standard deviation.
† Fisher exact test.
‡ Mann Whitney U test.

mouse FcγRIIb with remarkably similar binding strength to human FcγRIIb.[34] Therefore, human IgGs are most likely to bind to rat FcγRIIb. It should be noted that human mast cells and basophils express FcγRIIb. Thus, the results of the modified EXiLE test for measuring the ability of anti-IgE AAbs to induce FcεRI crosslinking using RS-ATL8 cells might reflect the ability of anti-IgE AAbs to induce FcεRI crosslinking using human mast cells and basophils.

The question is whether anti-FcεR1α AAbs detected in patients with CSU may play any physiological role, since the ability of these AAbs to induce aggregation of FcεRI did not differ significantly between the NC subjects and CSU patients (Fig. 2C). Addition of more than 3 ng/mL of CRA1 induced activation of basophils (Fig. 4A). CRA1 (3 ng/mL) induced about a 5-fold increase in the luciferase fluorescence intensity (Fig. 2A). However, a 5-fold increase induced by the anti-FcεRIα AAbs was observed in only two patients with CSU and none of NC subjects (Fig. 2C). These findings suggest that anti-FcεRIα AAbs obtained from a very small population of patients with CSU might be able to activate basophils.

The absence of any significant correlations between the results of ASST and the results of the modified EXiLE test (Table 3) suggested that circulating histamine-releasing factors may include not only IgG AAbs but also complement,[35] autoreactive IgE,[32,33]

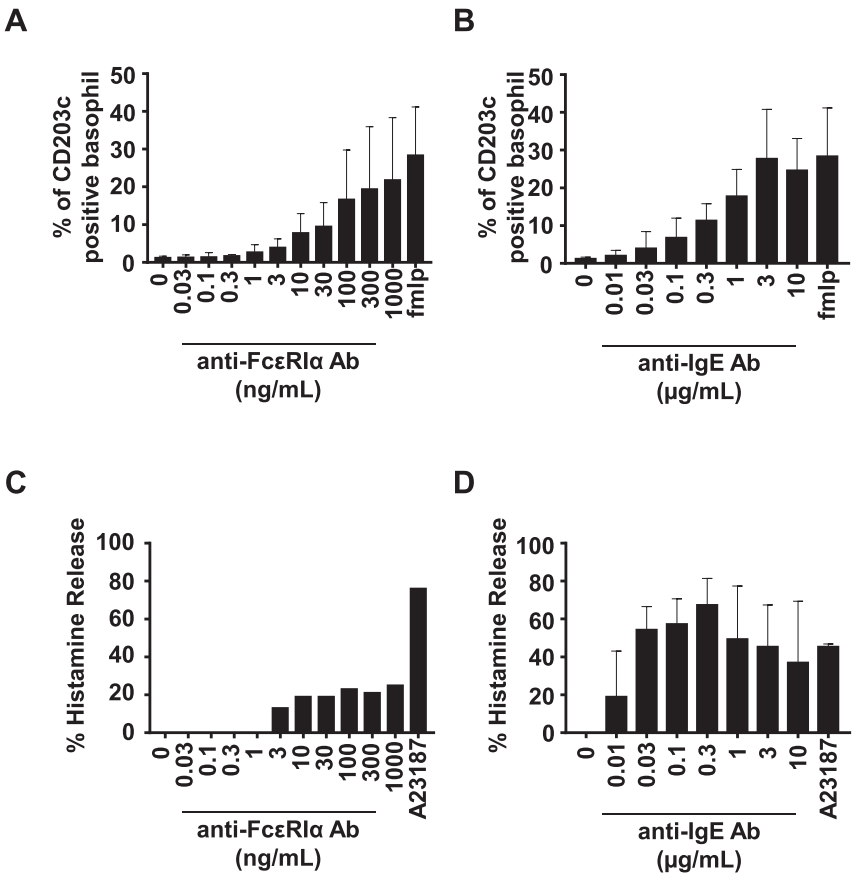

Fig. 4. Activation of human basophils and synovium-derived cultured mast cells following aggregation of FcεRI. The percentages of CD203c-positive basophils among all basophils after stimulation with 0.03–1000.00 ng/mL of mouse anti-human FcεRIα mAb (clone CRA1, **A**) and 0.01–10.00 μg/mL of rabbit anti-human IgE Ab **(B)** are shown. fmlp; N-formyl-methionyl-leucyl-phenylalanine (10^{-5} M) Results are shown as the means ± SD of three experiments using samples from three different healthy donors. Histamine release from human synovium-derived cultured mast cells in response to 0.03–1000.00 ng/mL of mouse anti-human FcεRIα mAb (clone CRA1, **C**) and 0.01–10.00 μg/mL of rabbit anti-human IgE Ab **(D)**. A23187: calcium ionophore A23187 (10^{-6} M) Results of **(D)** are shown as the mean ± SD of three experiments using three different patients with osteoarthritis. **(A)** representative data from three experiments using three different patients with osteoarthritis are shown in **(C)**.

neuropeptides,[36,37] coagulation factors[38] and unknown factors. A positive result of the ASST apparently persisted even after IgG depletion from the sera of patients with CSU, suggesting that a positive result of ASST may indicate the presence of other vasoactive factors rather than that of IgG AAbs.[39]

A significant difference in the duration of illness was noted between CSU patients with lower and higher concentrations of anti-IgE AAbs relative to the cutoff value (Table 2, $P = 0.0101$). The mechanisms have been unknown. Previous studies reported that the presence of serum anti-thyroid AAbs, levels of vitamin D, total IgE levels, and the present with concomitant angioedema or inducible urticaria were linked to disease duration.[40] Further research is needed to clarify the mechanisms why CSU patients with higher concentrations of anti-IgE AAbs may show longer disease duration.

Because the specificity revealed by the ROC analysis was only 50.9%, many NC subjects had higher titers than the tentative cutoff value. This was a limitation of the present study. We could not estimate a definite cutoff value for the ability of anti-IgE AAbs for aggregation of FcεRI because of the small number of patients and the paucity of previous reports suitable for verifying the validity of the cutoff value. The findings need to be confirmed in different cohorts, each comprising at least 200 individuals.

The result of this study is different from those published previously by researchers in the USA and Europe. They reported that the frequencies of patients, whose sera containing anti-FcεRIα AAbs have ability of degranulation of basophils, were higher compared with those, whose sera containing anti-IgE AAbs do[41–43] and that the frequencies of anti-FcεRIα AAbs-positive patients were higher than those of anti-IgE AAbs.[25,42,43] Several groups[6,9,11,13,41,42,44,45] have reported that the percentage of patients (or subjects) carrying anti-FcεRIα AAbs was higher in patients with CSU than in NC subjects. Although Fiebiger *et al.*[13] have reported that in patients with CSU the percentage of anti-IgE AAbs carrying patients (69%) was higher than the percentage of anti-FcεRIα AAbs carrying patients (37.5%), they also found anti-IgE AAbs in 73% and 26% of patients with atopic dermatitis and healthy controls, respectively and they found anti-FcεRIα AAbs neither in patients with atopic dermatitis nor healthy controls, suggesting that anti-IgE AAbs are poorly disease specific. A few have reported that the percentage of patients (or subjects) carrying anti-FcεRIα AAbs did not differ between CSU patients and NC subjects.[24,43,46] This discrepancy might be due to the different methods used for detecting the AAbs or differences in the populations examined. Furthermore, the finding that concentration of anti-IgE AAbs was significantly different between the NC subjects and CSU patients, whereas that of anti-FcεRIα AAbs was not, may be consistent with the clinical observation that omalizumab, a humanized anti-IgE mAb, was effective in most antihistamine-refractory CSU patients.[47] However, further studies are required to validate this hypothesis.

In conclusion, the ability of anti-IgE AAbs, compared with anti-FcεRIα AAbs, to induce FcεRI crosslinking was capable of

differentiating between NC subjects and patients with CSU. This finding provides further insight into the pathogenesis of CSU as an autoimmune disease.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2019.01.003.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

SI, ST, TE, KK, JK and TS performed experiments. SI, TE and KH contributed to data collection. SI, ST, TE and JK performed the statistical analysis. RN and HA advised experiments. YO designed the study and wrote the manuscript. SN supervised SI. TT and CR supervised SI, TE, ST and KH. All authors read and approved the final manuscript.

References

1. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA(2)LEN/EDF/WAO Guideline for the definition, classification, diagnosis and management of urticaria. The 2017 revision and update. *Allergy* 2018;**73**:1393–414.
2. Kaplan AP. Chronic spontaneous urticaria: pathogenesis and treatment considerations. *Allergy Asthma Immunol Res* 2017;**9**:477–82.
3. Church MK, Kolkhir P, Metz M, Maurer M. The role and relevance of mast cells in urticaria. *Immunol Rev* 2018;**282**:232–47.
4. Kolkhir P, Church MK, Weller K, Metz M, Schmetzer O, Maurer M. Autoimmune chronic spontaneous urticaria: what we know and what we do not know. *J Allergy Clin Immunol* 2016;**139**:1772–81.
5. Cho CB, Stutes SA, Altrich ML, Ardoin SP, Phillips G, Ogbogu PU. Autoantibodies in chronic idiopathic urticaria and nonurticarial systemic autoimmune disorders. *Ann Allergy Asthma Immunol* 2013;**110**:29–33.
6. Fiebiger E, Hammerschmid F, Stingl G, Maurer D. Anti-FcεRIα autoantibodies in autoimmune-mediated disorders. Identification of a structure-function relationship. *J Clin Invest* 1998;**101**:243–51.
7. Kikuchi Y, Kaplan AP. Mechanisms of autoimmune activation of basophils in chronic urticaria. *J Allergy Clin Immunol* 2001;**107**:1056–62.
8. Hide M, Francis DM, Grattan CE, Hakimi J, Kochan JP, Greaves MW. Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N Engl J Med* 1993;**328**:1599–604.
9. Ferrer M, Kinet JP, Kaplan AP. Comparative studies of functional and binding assays for IgG anti-FcεRIα (α-subunit) in chronic urticaria. *J Allergy Clin Immunol* 1998;**101**:672–6.
10. Niimi N, Francis DM, Kermani F, O'Donnell BF, Hide M, Kobza-Black A, et al. Dermal mast cell activation by autoantibodies against the high affinity IgE receptor in chronic urticaria. *J Invest Dermatol* 1996;**106**:1001–6.
11. Zuberbier T, Henz BM, Fiebiger E, Maurer D, Stingl G. Anti-FcεRIα serum autoantibodies in different subtypes of urticaria. *Allergy* 2000;**55**:951–4.
12. Kikuchi Y, Kaplan AP. A role for C5a in augmenting IgG-dependent histamine release from basophils in chronic urticaria. *J Allergy Clin Immunol* 2002;**109**:114–8.
13. Fiebiger E, Maurer D, Holub H, Reininger B, Hartmann G, Woisetschlager M, et al. Serum IgG autoantibodies directed against the α chain of FcεRI: a selective marker and pathogenetic factor for a distinct subset of chronic urticaria patients? *J Clin Invest* 1995;**96**:2606–12.
14. Nakamura R, Ishiwatari A, Higuchi M, Uchida Y, Nakamura R, Kawakami H, et al. Evaluation of the luciferase assay-based in vitro elicitation test for serum IgE. *Allergol Int* 2012;**61**:431–7.
15. Zuberbier T, Aberer W, Asero R, Bindslev-Jensen C, Brzoza Z, Canonica GW, et al. The EAACI/GA(2) LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: the 2013 revision and update. *Allergy* 2014;**69**:868–87.
16. Mathias SD, Crosby RD, Rosen KE, Zazzali JL. The minimal important difference for measures of urticaria disease activity: updated findings. *Allergy Asthma Proc* 2015;**36**:394–8.
17. Hide M, Hiragun T, Japanese Dermatological Association. Japanese guidelines for diagnosis and treatment of urticaria in comparison with other countries. *Allergol Int* 2012;**61**:517–27.
18. Konstantinou GN, Asero R, Maurer M, Sabroe RA, Schmid-Grendelmeier P, Grattan CE. EAACI/GA(2)LEN task force consensus report: the autologous serum skin test in urticaria. *Allergy* 2009;**64**:1256–68.
19. Ra C, Kuromitsu S, Hirose T, Yasuda S, Furuichi K, Okumura K. Soluble human high-affinity receptor for IgE abrogates the IgE-mediated allergic reaction. *Int Immunol* 1993;**5**:47–54.
20. Takai T, Yuuki T, Iwamoto-Yasue N, Okumura K, Ra C. Epitope analysis and primary structures of variable regions of anti-human FcεRI monoclonal antibodies, and expression of the chimeric antibodies fused with human constant regions. *Biosci Biotechnol Biochem* 2000;**64**:1856–67.
21. Takai T, Yuuki T, Ra C. Inhibition of IgE-dependent histamine release from human peripheral blood basophils by humanized Fab fragments that recognize the membrane proximal domain of the human FcεRI α-chain. *Int Arch Allergy Immunol* 2000;**123**:308–18.
22. Lee MF, Lin TM, Liu SW, Chen YH. A rapid method of detecting autoantibody against FcεRIα for chronic spontaneous urticaria. *PLoS One* 2014;**9**:e109565.
23. Mozena JD, Tinana A, Negri J, Steinke JW, Borish L. Lack of a role for cross-reacting anti-thyroid antibodies in chronic idiopathic urticaria. *J Invest Dermatol* 2010;**130**:1860–5.
24. Pachlopnik JM, Horn MP, Fux M, Dahinden M, Mandallaz M, Schneeberger D, et al. Natural anti-FcεRIα autoantibodies may interfere with diagnostic tests for autoimmune urticaria. *J Autoimmun* 2004;**22**:43–51.
25. Staubach P, Onnen K, Vonend A, Metz M, Siebenhaar F, Tschentscher I, et al. Autologous whole blood injections to patients with chronic urticaria and a positive autologous serum skin test: a placebo-controlled trial. *Dermatology* 2006;**212**:150–9.
26. Atta AM, Rodrigues MZ, Sousa CP, Medeiros Junior M, Sousa-Atta ML. Autoantibody production in chronic idiopathic urticaria is not associated with Helicobacter pylori infection. *Braz J Med Biol Res* 2004;**37**:13–7.
27. Gruber BL, Baeza ML, Marchese MJ, Agnello V, Kaplan AP. Prevalence and functional role of anti-IgE autoantibodies in urticarial syndromes. *J Invest Dermatol* 1988;**90**:213–7.
28. Nakamura R, Uchida Y, Higuchi M, Nakamura R, Tsuge I, Urisu A, et al. A convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression in cultured mast cells. *Allergy* 2010;**65**:1266–73.
29. Eskandari N, Bastan R, Ahmadi M, Peachell PT. Evaluation of the correlation and reproducibility between histamine, IL-4, and IL-13 release from human basophils. *Ir J Allergy Asthma Immunol* 2014;**13**:190–7.
30. Zenarruzabeitia O, Vitalle J, Terren I, Orrantia A, Astigarraga I, Dopazo L, et al. CD300c co-stimulates IgE-mediated basophils activation and its expression is increased in cow s milk allergy. *J Allergy Clin Immunol* 2019;**143**:700–11.e5.
31. De Swerdt A, Van Den Keybus C, Kasran A, Cadot P, Neyens K, Coorevits L, et al. Detection of basophil-activating IgG autoantibodies in chronic idiopathic urticaria by induction of CD 63. *J Allergy Clin Immunol* 2005;**116**:662–7.
32. Hatada Y, Kashiwakura J, Hayama K, Fujisawa D, Sasaki-Sakamoto T, Terui T, et al. Significantly high levels of anti-dsDNA immunoglobulin E in sera and the ability of dsDNA to induce the degranulation of basophils from chronic urticaria patients. *Int Arch Allergy Immunol* 2013;**161**(Suppl 2):154–8.
33. Schmetzer O, Lakin E, Topal FA, Preusse P, Freier D, Church MK, et al. IL-24 is a common and specific autoantigen of IgE in chronic spontaneous urticaria. *J Allergy Clin Immunol* 2018;**142**:876–82.
34. Dekkers G, Bentlage AEH, Stegmann TC, Howie HL, Lissenberg-Thunnissen S, Zimring J, et al. Affinity of human IgG subclasses to mouse Fc γ receptors. *MAbs* 2017;**9**:767–73.
35. Ferrer M, Nakazawa K, Kaplan AP. Complement dependence of histamine release in chronic urticaria. *J Allergy Clin Immunol* 1999;**104**:169–72.
36. Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, et al. Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. *J Allergy Clin Immunol* 2014;**134**:622–33. e9.
37. Metz M, Krull C, Hawro T, Saluja R, Groffik A, Stanger C, et al. Substance P is upregulated in the serum of patients with chronic spontaneous urticaria. *J Invest Dermatol* 2014;**134**:2833–6.
38. Asero R, Tedeschi A, Riboldi P, Cugno M. Plasma of patients with chronic urticaria shows signs of thrombin generation, and its intradermal injection causes wheal-and-flare reactions much more frequently than autologous serum. *J Allergy Clin Immunol* 2006;**117**:1113–7.
39. Fagiolo U, Kricek F, Ruf C, Peserico A, Amadori A, Cancian M. Effects of complement inactivation and IgG depletion on skin reactivity to autologous serum in chronic idiopathic urticaria. *J Allergy Clin Immunol* 2000;**106**:567–72.

40. Deza G, Ricketti PA, Gimenez-Arnau AM, Casale TB. Emerging biomarkers and therapeutic pipelines for chronic spontaneous urticaria. *J Allergy Clin Immunol Pract* 2018;**6**:1108–17.
41. Tong LJ, Balakrishnan G, Kochan JP, Kinet JP, Kaplan AP. Assessment of autoimmunity in patients with chronic urticaria. *J Allergy Clin Immunol* 1997;**99**:461–5.
42. Sabroe RA, Fiebiger E, Francis DM, Maurer D, Seed PT, Grattan CE, et al. Classification of anti-FcεRI and anti-IgE autoantibodies in chronic idiopathic urticaria and correlation with disease severity. *J Allergy Clin Immunol* 2002;**110**:492–9.
43. Eckman JA, Hamilton RG, Gober LM, Sterba PM, Saini SS. Basophil phenotypes in chronic idiopathic urticaria in relation to disease activity and autoantibodies. *J Invest Dermatol* 2008;**128**:1956–63.
44. Sun L, Erxun K, Li J, Yang J, Han C. Correlations between anti-mast cell autoantibodies and chronic idiopathic urticaria. *Ann Dermatol* 2014;**26**:145–9.
45. Hidvegi B, Nagy E, Szabo T, Temesvari E, Marschalko M, Karpati S, et al. Correlation between T-cell and mast cell activity in patients with chronic urticaria. *Int Arch Allergy Immunol* 2003;**132**:177–82.
46. Vasagar K, Vonakis BM, Gober LM, Viksman A, Gibbons Jr SP, Saini SS. Evidence of in vivo basophil activation in chronic idiopathic urticaria. *Clin Exp Allergy* 2006;**36**:770–6.
47. Saini SS, Bindslev-Jensen C, Maurer M, Grob JJ, Bulbul Baskan E, Bradley MS, et al. Efficacy and safety of omalizumab in patients with chronic idiopathic/spontaneous urticaria who remain symptomatic on H1 antihistamines: a randomized, placebo-controlled study. *J Invest Dermatol* 2015;**135**:925.