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Original Article

A novel detection method for cross-linking of IgE-receptors by autoantibodies in chronic spontaneous urticaria

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AAbs, autoantibodies; Alpha, amplified luminescence proximity homogeneous assay; ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; EXiLE, IgE crosslinking-induced luciferase expression; FBS, fetal bovine serum; FcεRIα, high-affinity IgE receptor alpha subunit; HRT, histamine release test; HSA, human serum albumin; N.D., not detected; N.P., not performed; nos., numbers; PBS-T, phosphate-buffered saline containing 0.05% Tween-20; RT, room temperature

ABSTRACT

Background: Autoantibodies (AAbs) against immunoglobulin E (IgE) antibodies (Abs) and their high-affinity receptor alpha subunits (FcεRIα) are key factors in the elicitation of type IIb autoimmune chronic spontaneous urticaria (type IIb aiCSU). In this study, we aimed to develop a new method to detect functional anti-FcεRIα and anti-IgE AAbs, which can crosslink the plural FcεRIα molecules and IgE Abs on the surface of mast cells and basophils, in sera from aiCSU patients using the amplified luminescence proximity homogeneous assay (Alpha).

Methods: Sera were obtained from 14 aiCSU patients, as diagnosed by recurrent chronic spontaneous urticaria episodes and positive results for the autologous serum skin test and/or histamine release test (HRT). The AAbs to FcεRIα and IgE Abs were determined in sera from aiCSU patients using enzyme-linked immunosorbent assay (ELISA) and Alpha by cross-linking (AlphaCL) of IgE Abs and/or FcεRIα.

Results: Serum anti-FcεRIα and anti-IgE AAb levels were not significantly different between aiCSU patients and healthy subjects in ELISA. Anti-FcεRIα AAbs were detected in 10 of 14 aiCSU patients who displayed positive (5/5) and negative (5/9) results in the HRT for anti-FcεRIα AAbs by AlphaCL, whereas no signals were observed in healthy subjects. Additionally, anti-IgE AAbs were detected in two of four aiCSU patients who displayed positive results in the HRT for anti-IgE AAbs.

Conclusions: A new assay method using AlphaCL can detect anti-FcεRIα and anti-IgE AAbs with FcεRIα- and IgE-crosslinking abilities in sera from aiCSU patients. This simple and practical assay method may be available as a diagnostic tool for urticaria patients.

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Introduction

Chronic spontaneous urticaria (CSU) is defined as a skin disorder characterized by the recurrences of wheals and/or angioedema for at least 6 weeks with no specific eliciting factor.¹ Although the underlying mechanisms of CSU pathogenesis are not fully understood, mast cell and basophil degranulation caused by autoantibodies (AAbs) is considered as a key factor in its elicitation.² CSU caused by AAb-mediated reaction is defined as autoimmune CSU

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(aiCSU). The aiCSU has been divided into two endotypes: type I aiCSU and type II aiCSU. In type I aiCSU, patients have immunoglobulin E (IgE) AAbs against autoantigens, including thyroperoxidase,^{3–6} double-stranded DNA,^{6,7} interleukin-24,^{5,8,9} tissue factor¹⁰ and thyroglobulin.⁵ Type II aiCSU patients possess IgG AAbs bound to IgE antibodies (Abs) and/or the high-affinity IgE receptor alpha subunit (FcεRIα) on the surface of mast cells and basophils.^{1,11–15} These studies suggested that some CSU symptoms can be elicited by the crosslinking of plural FcεRI molecules on the surface of mast cells and basophils with autoantigens, anti-IgE and/or anti-FcεRIα AAbs.

The detection of functional anti-FcεRIα and/or anti-IgE AAbs in sera has been proposed as a screen for type II aiCSU. In clinical settings, the autologous serum skin test (ASST) is frequently performed to screen for AAbs in type II aiCSU patients because of its simplicity. However, the ASST can generate false-positive results because of the presence of some histamine-inducing factors, including bradykinin,¹⁶ C5a¹⁷ and low-molecular-weight mast cell-activating factors.¹⁸ The histamine release test (HRT) and the basophil activation test are also performed to detect functional AAbs against FcεRIα and/or IgE Abs. However, basophils from healthy donors are needed to perform these tests, and the degree of IgE- or FcεRIα-mediated basophil activation varies among basophil donors.^{15,19} To overcome these problems, Izaki *et al.* developed the luciferase-reporting mast cell line RS-ATL8, which expresses human FcεRI, to detect IgE crosslinking-induced luciferase expression (EXiLE) to determine mast cell activation by anti-FcεRIα and anti-IgE AAbs from CSU patients.¹⁹ The AAbs in CSU patients' sera are also detected using immunoblot analysis and enzyme-linked immunosorbent assay (ELISA). However, their specificities were insufficient to diagnose type II aiCSU because AAbs without degranulation-inducing activity were frequently detected in subjects without CSU.^{20,21} Therefore, there is an unmet need for a highly specific, simple and practical in vitro assay to diagnose type II aiCSU.

Amplified luminescence proximity homogeneous assay (Alpha) technology is a bead-based proximity assay used to detect an intermolecular interaction with high sensitivity and specificity in a biological sample.^{22,23} In this study, we developed a simple, highly specific and practical in vitro assay method to detect AAbs in CSU based on Alpha involving cross-linking (AlphaCL) of IgE Abs and receptors (Fig. 1).

Methods

Patients

We enrolled 25 CSU patients, as diagnosed by the EAACI/GA2LEN/EDF/WAO guideline for urticaria, from Hiroshima University Hospital and Shimane University Hospital between August 2002 and April 2020. Among these patients, we used sera collected from fourteen patients who displayed positive results for the ASST and/or HRT in this study. The characteristics of the patients are summarized in Table 1. Sera from nine healthy subjects were used as controls. The sera were stored at –80 °C until use. This study was approved by the ethics committee of Hiroshima University (approval No. E–1968-1) in compliance with the Declaration of Helsinki and current legal regulations in Japan. Written informed consent was obtained from the patients or we provided the opportunity to opt-out.

Autologous serum skin test (ASST)

The ASST was performed according to the method reported by Takahagi *et al.*^{14,24} Briefly, 50 µL patient autologous serum was intradermally injected into the patient's forearm. Physiologic saline (50 µL) and histamine solution (20 µL, 10 µg/mL in saline) were simultaneously injected intradermally as negative and positive controls, respectively. The responses were measured at 30 min after injections. When the serum-induced wheal displayed a diameter of 1.5 mm greater than the saline control, we registered a result as positive for patients who took no H₁-antihistamines. In the case of patients with an H₁-antihistamine intake, we considered a result as positive when the serum-induced flare diameter was 5 mm greater than the saline control or larger than that induced by histamine solution.

Histamine release test (HRT)

The HRT was performed according to the method described by Matsuo *et al.*²⁵ with a slight modification. Briefly, peripheral blood leukocytes collected from two healthy volunteers were suspended with buffer A (137 mM NaCl, 5 mM glucose, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.03% human serum albumin [HSA]), and the suspension

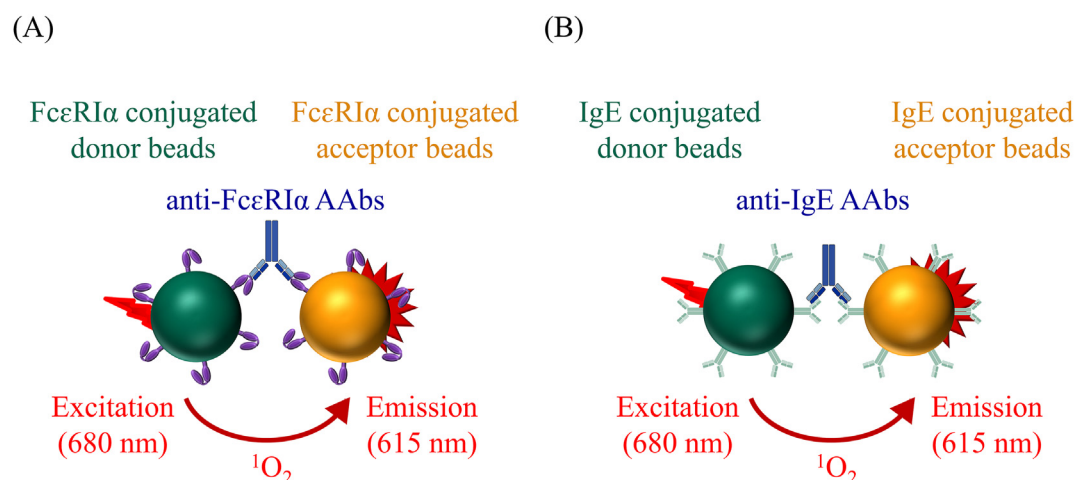


Fig. 1. The principles of AlphaCL to detect anti-FcεRIα (A) and anti-IgE AAbs (B). When FcεRIα- or IgE-conjugated acceptor and donor beads come into proximity by cross-linking with the respective AAb, singlet oxygen is released from donor beads by excitation at 680 nm. The released singlet oxygen triggers an energy transfer cascade in the acceptor beads, resulting in a sharp emission peak at 615 nm.

Table 1
Characteristics of aiCSU patients.

No.	Age (years)	Gender	ASST	HRT (%)		ELISA (Absorbance)	
				Lactic acid treated	Lactic acid untreated	anti-FcεRIα IgG	anti-IgE IgG
Patients							
1	66	F	Positive	71.0 [†]	18.0 [†]	0.06	0.18
2	77	F	Positive	66.2 [‡]	21.6 [‡]	0.11	1.24
3	38	F	Negative	17.5 [§]	0.4 [§]	0.10	0.41
4	42	F	Positive	45.4 [¶]	30.4 [¶]	0.41	0.83
5	70	F	N.P.	38.3 [#]	28.9 [#]	0.09	0.18
6	58	M	Positive	0 [†]	27.0 [†]	0.12	0.52
7	76	F	Positive	0 [§]	43.2 [§]	0.18	1.32
8	53	F	Positive	0 [§]	15.2 [§]	0.07	0.13
9	73	M	Positive	6.4 [‡]	33.0 [‡]	0.24	0.38
10	32	F	Positive	0 [§]	3.1 [§]	0.23	0.17
11	38	F	Positive	1.2 [§]	3.0 [§]	0.05	0.13
12	69	F	Positive	1.6 [§]	1.5 [§]	0.18	0.17
13	45	M	Positive	0.5 [§]	0.3 [§]	0.23	0.18
14	30	F	Positive	0.6 [§]	0 [§]	0.18	0.43
Healthy subjects							
1	—	—	—	—	—	0.22	0.15
2	—	—	—	—	—	0.13	0.28
3	—	—	—	—	—	0.09	0.07
4	—	—	—	—	—	0.29	0.35
5	—	—	—	—	—	0.11	0.29
6	—	—	—	—	—	0.16	0.76
7	—	—	—	—	—	0.07	0.21
8	—	—	—	—	—	0.06	0.36
9	—	—	—	—	—	0.16	0.15

Each HRT was performed using leukocytes from two healthy donors. The results are shown as histamine release (%) from the donor's leukocytes (each symbol) that showed the highest histamine release induced by the patients' sera. When each donor leukocyte untreated with lactic acid was stimulated with anti-IgE Abs, histamine release was [†]62.0%, [‡]30.6%, [§]54.6%, [¶]59.4% and [#]70.7%, respectively. ASST, autologous serum skin test; HRT, histamine release test; AAbs, autoantibodies; M/F, male/female; N.P., not performed.

was divided into two equal volumes. Following centrifugation of each suspension at 450×g for 15 min, one pellet was resuspended with saline containing 0.03% HSA and 0.1% lactic acid to remove IgE Abs on the surface of basophils. Following centrifugation, each pellet containing basophils with or without the IgE Abs removed was resuspended with buffer A containing 2 mM CaCl₂ and 1 mM MgCl₂. Either each patient's serum or rabbit anti-human IgE antibodies (Dako, Santa Clara, CA, USA) was added to each of the basophil suspensions simultaneously. Following incubation for 40 min at 37 °C, the suspensions were diluted with buffer A and centrifuged at 1400×g for 5 min. The supernatants were transferred to new tubes and the cell pellets were resuspended with buffer A without HSA. Subsequently, 20% HClO₄ solution was added to both supernatants and cell suspensions, and incubated for 30 min at room temperature (RT). Following centrifugation of these samples at 20,000×g for 10 min, the amount of histamine was determined by high performance liquid chromatography as described by Matsuo *et al.*²⁵ Net histamine release was calculated as the percentage of the total amount of histamine in the cells after subtraction of spontaneous release. A positive result was defined as a net histamine release ≥5%. When the IgE Abs are removed from the surface of basophils by treatment with lactic acid, anti-FcεRIα AAb binds to FcεRIα more easily than basophils untreated because steric hindrance of IgE Abs may be reduced. In this case, anti-IgE AAbs-mediated histamine release is not increased. Thus, when the histamine release from lactic acid-treated basophils was greater than that from untreated cells, we defined this as “anti-FcεRIα AAbs”. In the opposite case, we defined this as “anti-IgE AAbs”.

Measurement of serum anti-FcεRIα and anti-IgE AAb levels by ELISA

ELISA was performed to detect IgG AAbs against FcεRIα or IgE Abs as described previously by Altrichter *et al.*²⁶ with a slight

modification. Briefly, microplate wells (F8 MaxiSorp loose Nunc-Immuno Modules, Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated with 200 μL recombinant human FcεRIα (500 ng/mL, R&D SYSTEM, Minneapolis, MN, USA) or native human IgE Ab (2.5 μg/mL, abcam, Cambridge, UK) dissolved in phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C. Following washing with PBS containing 0.05% Tween-20 (PBS-T) five times, the plates were incubated with PBS containing 10% fetal bovine serum (FBS) in PBS for 1 h at RT. Following washing with PBS-T, 200 μL of each sera sample (diluted 1:1000 with 10% FBS) was added to each well and incubated for 2 h at RT. Following washing with PBS-T, the wells were incubated with 200 μL horseradish peroxidase-conjugated goat anti-human IgG Abs (Thermo Fisher Scientific) (diluted 1:10000 with 10% FBS) for 1 h at RT. The wells were washed with PBS-T and incubated with 100 μL 3,3',5,5'-tetramethylbenzidine solution (KPL, Gaithersburg, MD, USA) at RT. After a 15-min incubation, the reaction was terminated with 100 μL 1 M phosphoric acid. Absorbance was determined at 450 nm against 620 nm as a reference using a Multiskan GO (Thermo Fisher Scientific).

Detection of functional anti-FcεRIα and anti-IgE AAbs using AlphaCL

To detect functional anti-FcεRIα or anti-IgE AAbs in sera, we prepared donor and acceptor beads (each 5 mg/mL) conjugated with FcεRIα or IgE Ab proteins according to the manufacturer's protocol (PerkinElmer, Inc., Waltham, MA, USA). Figure 1 shows the principles of our assay methods using Alpha technology to detect the crosslinking of FcεRIα or IgE Abs by AAbs. When FcεRIα- or IgE-conjugated acceptor and donor beads come into proximity by crosslinking with the respective AAb, singlet oxygen is released from donor beads by excitation at 680 nm. The released singlet oxygen triggers an energy transfer cascade in the acceptor beads, resulting in a sharp emission peak at 615 nm. Therefore, we can

quantitatively determine the FcεRIα- or IgE-crosslinking AAb levels in sera from aiCSU patients by measuring their emission peak signal counts. The beads were diluted with immunoassay buffer (PerkinElmer, Inc.) and mixed at appropriate concentrations. To optimize anti-FcεRIα or anti-IgE AAb detection, we evaluated the incubation time, bead concentrations and serum dilution. To determine the incubation time and bead concentrations, 20 μL of each bead mixture (acceptor, 5–20 μg/mL; donor, 5–20 μg/mL) and 20 μL of each patient's serum (final 4% serum from patient no. 2 and 1% serum from patient no. 6 for anti-FcεRIα and anti-IgE AAb detection, respectively) diluted with immunoassay buffer were added to 1/2 AreaPlate-96 culture plates (PerkinElmer, Inc.) and were incubated for 1–24 h at RT in the dark. To determine the serum dilution, 20 μL of each bead mixture (acceptor, 20 μg/mL; donor, 5 μg/mL for anti-FcεRIα AAbs or 20 μg/mL for anti-IgE AAbs) was incubated with 20 μL of patient's serum (final 0.25%–8%) for 1 h (anti-FcεRIα AAbs) or 24 h (anti-IgE AAbs) at RT in the dark.

To detect anti-FcεRIα or anti-IgE AAbs in aiCSU patients' sera, 20 μL of each bead mixture (acceptor, 20 μg/mL and donor, 5 μg/mL for anti-FcεRIα AAbs or 20 μg/mL for anti-IgE AAbs) was incubated with 20 μL serum from healthy subjects or patients (final 4% and 1% for anti-FcεRIα and anti-IgE AAbs, respectively) for 1 h (anti-FcεRIα AAbs) or 24 h (anti-IgE AAbs) at RT in the dark. As positive controls for anti-FcεRIα or anti-IgE AAbs, 20 μL of 4% or 1% pooled human serum (Sigma–Aldrich, St. Louis, MO, USA) containing a mouse anti-human FcεRIα mAb (CRA1, 0.005–1 μg/mL, Bio Academia, Osaka, Japan), which binds to FcεRIα in a non-IgE competitive manner, or a rabbit anti-human IgE Ab (0.005–1 μg/mL, Dako) were used instead of patients' serum, respectively. For the inhibition study, patients' sera (8% for anti-FcεRIα AAbs and 2% for anti-IgE AAbs), which was pre-incubated with FcεRIα or IgE proteins (10 μg/mL each) for 1 h at RT, was used. Measurement was performed using a multimode plate reader (EnSpire™, PerkinElmer, Inc.). The Alpha signal (counts) was calculated as net counts by subtracting the counts in buffer alone from the total counts in each sample.

Statistics

Data were displayed as the means ± standard deviations of the mean (S.D.). Mean value differences between groups were assessed using Student's t-tests. Strength of the correlations in the results between ELISA and the HRT and between ELISA and AlphaCL were analyzed by Spearman's correlation coefficient by rank test. $P < 0.05$ was considered statistically significant.

Results

Measurement of anti-FcεRIα and anti-IgE AAbs in sera from aiCSU patients using the HRT and ELISA

To detect anti-FcεRIα and anti-IgE AAbs in sera from 14 aiCSU patients, the HRT and ELISA were performed. Sera from 9 of 14 aiCSU patients showed positive results in the HRT using the leukocytes of healthy donors either untreated or treated with lactic acid (Table 1). Among these nine patients, sera from five patients (nos. 1–5) released greater histamine from lactic acid-treated leukocytes than that from untreated cells, whereas the other patients (nos. 6–9) displayed the opposite result. These results suggest that the patients nos. 1–5 and nos. 6–9 had anti-FcεRIα AAbs and anti-human IgE AAbs, respectively.

In the ELISA, serum levels of anti-FcεRIα (0.16 ± 0.10 AU for patients and 0.14 ± 0.07 AU for healthy subjects; $P = 0.666$) and anti-IgE AAbs (0.45 ± 0.40 AU for patients and 0.29 ± 0.20 AU for healthy subjects; $P = 0.300$) were not significantly different

between the aiCSU patients and healthy subjects (Table 1). In this study, no statistical correlations were obtained by Spearman's correlation coefficient by rank test between the serum levels of AAbs for FcεRIα or IgE Abs in the ELISA and their responses in the HRT among CSU patients.

Measuring conditions for anti-FcεRIα AAbs by AlphaCL

To optimize the detection conditions for anti-FcεRIα AAbs using the Alpha system, we first examined the optimal incubation time and the concentrations of acceptor and donor beads conjugated with FcεRIα by using the serum from one patient (no. 2). Following a 1-h incubation, the signals were readily detected and gradually increased until 24 h (Fig. 2A). The signal count was the highest when the acceptor and donor beads were used at the concentrations of 20 μg/mL and 5 μg/mL, respectively. We further examined the optimal serum dilutions for the Alpha system by a 1-h incubation of acceptor (20 μg/mL) and donor (5 μg/mL) beads with sera from three patient (nos. 2, 3 and 5). The signal count was enhanced by adding serum in a concentration-dependent manner among three patients (Fig. 2B). When the serum was added at 4%, the signal count exceeded that of the buffer control by more than two S.D. Therefore, for further studies, we detected anti-FcεRIα AAbs in 4% sera using 20 μg/mL acceptor and 5 μg/mL donor beads in a 1-h incubation at RT. An inhibition study was performed to confirm the specific crosslinking of FcεRIα proteins between those on the surfaces of acceptor and donor beads with anti-FcεRIα AAbs from aiCSU patients (Fig. 2C). The addition of FcεRIα protein to the patients' sera completely suppressed the Alpha signals, indicating that anti-FcεRIα AAbs in patients' sera specifically crosslink plural FcεRIα proteins on the surface of acceptor and donor beads. Finally, we tested the concentration-dependent crosslinking of acceptor and donor beads using a mouse anti-human FcεRIα mAb (CRA1). As shown in Figure 2D, the signal counts were increased by CRA1 in a concentration-dependent manner within 0.005–1 μg/mL.

Measuring conditions for anti-IgE AAbs by AlphaCL

As with the detection of anti-FcεRIα AAbs, we attempted to determine the optimal incubation time and bead and serum concentrations to detect anti-IgE AAbs using the Alpha system. The highest signal count was obtained by a 24-h incubation with 20 μg/mL of both the acceptor and donor beads with patient serum (no. 6) (Fig. 3A). While the signal count in 1% serum was higher than that in 0.25%, this elevated count decreased with serum concentrations of 4 and 8% (Fig. 3B). Therefore, for further studies, we detected anti-IgE AAbs in 1% sera using 20 μg/mL acceptor and 20 μg/mL donor beads in a 24-h incubation at RT. The addition of IgE Abs to the patients' sera suppressed the signal counts, suggesting that the anti-IgE AAbs in the patients' sera were most specifically crosslinking IgE molecules between those on the surfaces of acceptor and donor beads (Fig. 3C). The signal intensity was increased by a rabbit anti-human IgE Ab in a concentration-dependent manner within 0.005–1 μg/mL (Fig. 3D).

Detection of anti-FcεRIα and anti-IgE AAbs in aiCSU patients using AlphaCL

We attempted to detect the functional anti-FcεRIα and anti-IgE AAbs with FcεRIα and IgE-crosslinking abilities, respectively, in healthy subjects and aiCSU patients using AlphaCL. Signal counts for anti-FcεRIα AAbs were observed in 10 of 14 (71.4%) aiCSU patients, whereas no signals were observed in all healthy subjects (Fig. 4A). Functional anti-FcεRIα AAbs were detected in all patients

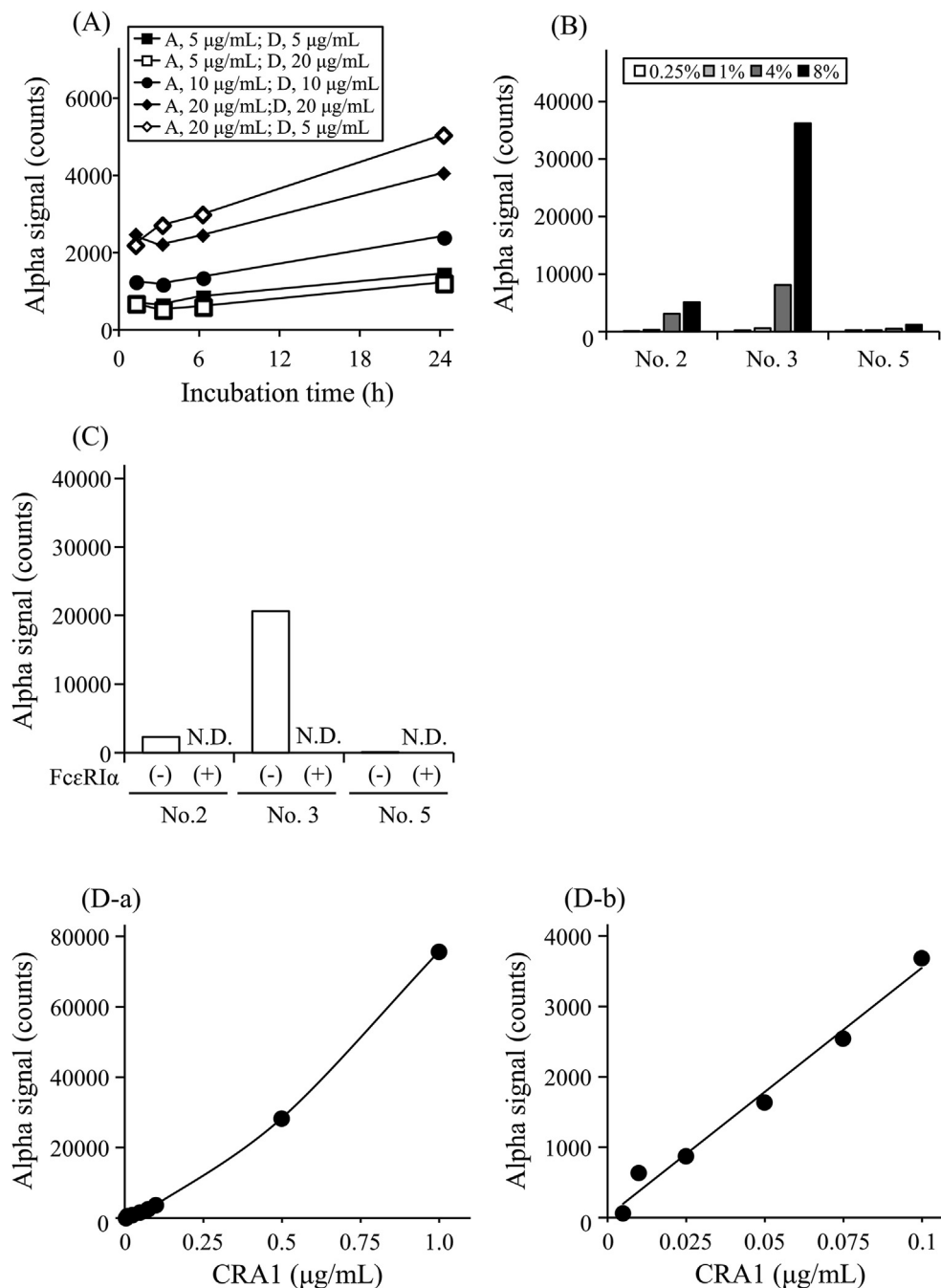


Fig. 2. Optimization of AlphaCL to detect anti-FcεRIα AAbs. (A) Determination of optimal acceptor and donor bead concentrations and incubation time using serum from aiCSU patient no. 2. A, acceptor bead concentrations; D, donor bead concentrations. (B) Determination of optimal dilutions of sera from three aiCSU patients. (C) Specific crosslinking of FcεRIα proteins on the surface of acceptor and donor beads by anti-FcεRIα AAbs in the sera from three aiCSU patients. N.D., not detected. (D) Concentration-dependent crosslinking of acceptor and donor beads by mouse anti-human FcεRIα mAb (CRA1) over the entire range (D-a, 0.005–1 μg/mL) and at low concentrations (D-b, 0.005–0.1 μg/mL). The results show typical data from one of two separate experiments.

who had anti-FcεRIα AAbs (nos. 1–5) and one of four patients who had anti-IgE AAbs (nos. 6–9) based on the results of the HRT. Functional anti-FcεRIα AAbs were also detected in four of five patients who displayed negative results in the HRT (nos. 10–14) in AlphaCL. Signal counts were obtained in two of four patients who had anti-IgE AAbs (nos. 6–9) (Fig. 4B). Among two patients, one patient (no. 7) showed positive results in AlphaCL for both anti-FcεRIα and anti-IgE AAbs (Fig. 4). The AlphaCL system for the detection of functional anti-FcεRIα and anti-IgE AAbs in aiCSU patient serum displayed higher sensitivity (11/14, 78.6%) compared to

that of the HRT (9/14, 64.2%). The AlphaCL signals for anti-FcεRIα AAbs ($P = 0.044$) and anti-IgE AAbs ($P = 0.013$) were significantly higher in the HRT-positive patients than those in the HRT-negative patients.

In this study, no statistical correlation was obtained by Spearman's correlation coefficient by rank test between the serum levels of anti-FcεRIα AAbs in the ELISA and their AlphaCL signals among CSU patients. On the other hand, we could not analyze the correlation for anti-IgE AAbs statistically because only two patients showed positive results in AlphaCL.

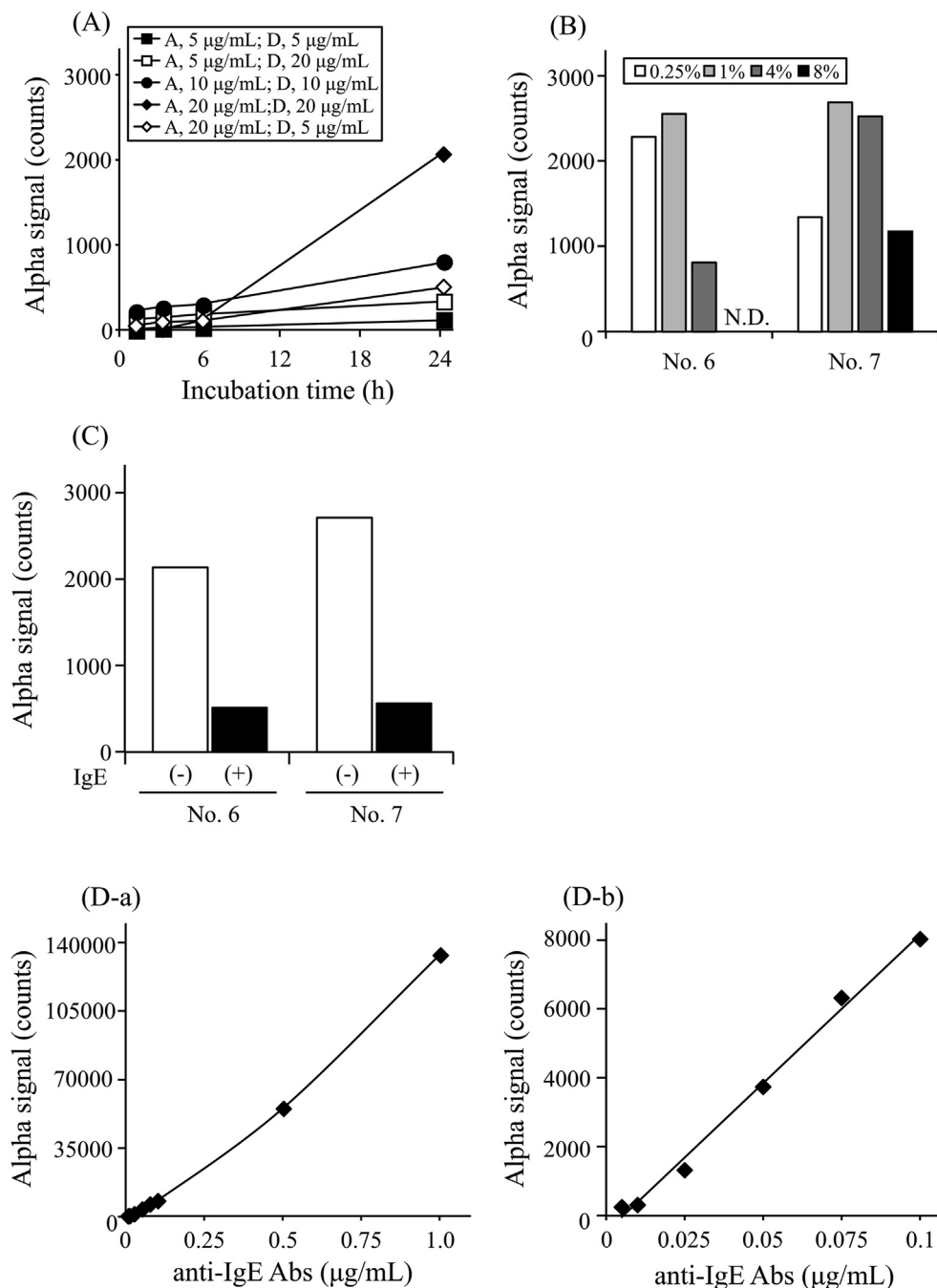


Fig. 3. Optimization of AlphaCL to detect anti-IgE AAbs. (A) Determination of optimal acceptor and donor bead concentrations and incubation time using serum from aiCSU patient no. 6. A, acceptor bead concentrations; D, donor bead concentrations. (B) Determination of optimal dilutions of sera from two aiCSU patients. N.D., not detected. (C) Specific crosslinking of IgE Abs on the surface of acceptor and donor beads by anti-IgE AAbs in the sera from two aiCSU patients. (D) Concentration-dependent crosslinking of acceptor and donor beads by rabbit anti-human IgE Ab over the entire range (D-a, 0.005–1 µg/mL) and at low concentrations (D-b, 0.005–0.1 µg/mL). The results show typical data from one of two separate experiments.

Discussion

Many studies have reported that sera AAb detection can be utilized to screen for type IIb aiCSU.^{12,14,19,26–31} However, there was previously no simple, highly specific or practical assay method to detect functional AAbs that crosslink plural FcεRI on the surface of mast cells and basophils in clinical settings. In this study, we established a new assay method to detect functional anti-FcεRIα and anti-IgE AAbs in sera from aiCSU patients using AlphaCL. Our new assay method displayed higher sensitivity for

the detection of functional anti-FcεRIα and anti-IgE AAbs in aiCSU patient serum compared to that of the HRT, as shown by the detection of functional anti-FcεRIα AAbs in sera from 11 of 14 (78.6%) aiCSU patients, whereas some of them had a negative result in the HRT (Fig. 4). Furthermore, this assay method provides the following four benefits in comparison with conventional assays: 1) use of non-basophils from healthy donors or cultured mast cells, 2) highly specific detection for functional AAbs against FcεRIα and IgE Abs, 3) feasible in any facility and 4) minimally invasive for subjects.

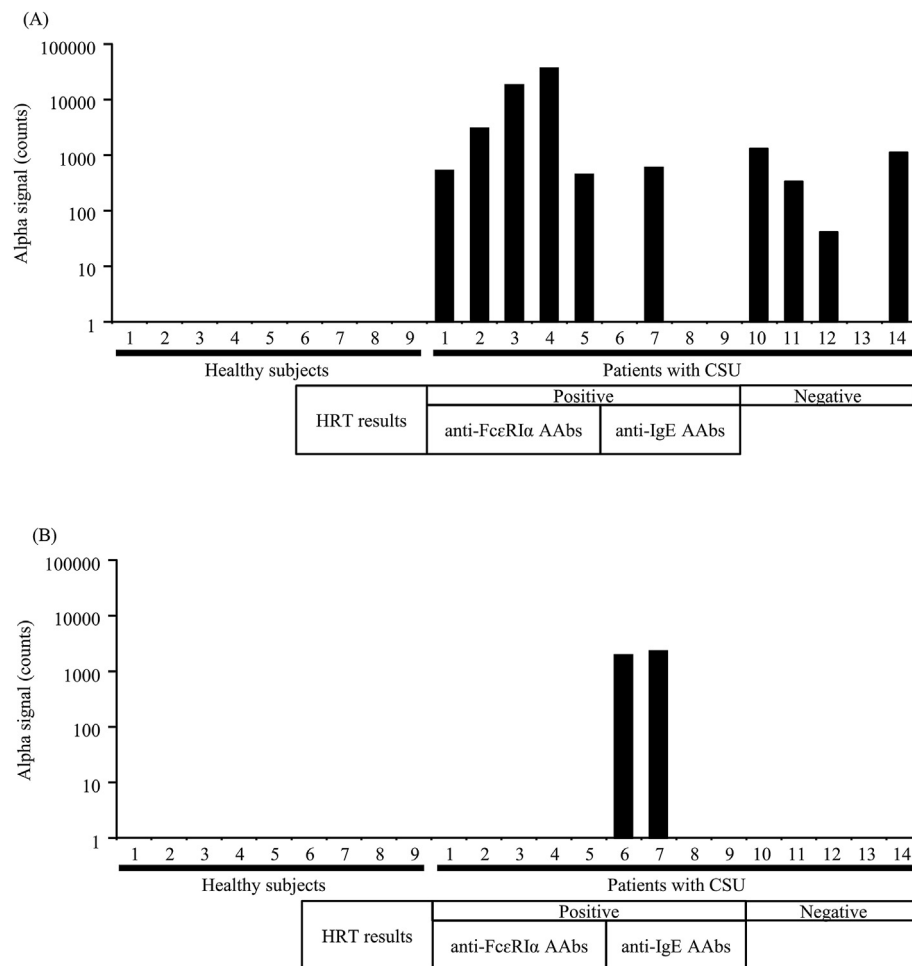


Fig. 4. Detection of anti-FcεRIα AAbs (A) or anti-IgE AAbs (B) in sera from healthy subjects and aiCSU patients by AlphaCL. When the histamine release from lactic acid-treated leukocytes in the HRT was greater than that from untreated cells (in patient nos.1–5), we described this as “anti-FcεRIα AAbs”. In the opposite case (in patient nos. 6–9), we described this as “anti-IgE AAbs”. “Negative” means histamine release rates were <5% from both untreated and lactic acid-treated leukocytes.

The AlphaCL signal counts for the detection of anti-FcεRIα and anti-IgE AAbs were increased by CRA1 and a rabbit anti-human IgE Ab, respectively, in a concentration-dependent manner (Fig. 2D, 3D). The results demonstrated that good correlations were obtained between the signal counts and the CRA1 or anti-IgE Abs concentration in the ranges of 0.005–1 μg/mL. Izaki *et al.* reported that the anti-FcεRIα and anti-IgE AAb concentrations in the purified IgG fraction from CSU patients were ~1.9–2.3 and ~1.2–1.4 μg/mL, respectively.¹⁹ In their study, the total IgG concentration in purified fractions decreased to one-sixth of those in the original sera.¹⁹ Therefore, the anti-FcεRIα and anti-IgE AAb concentrations assayed in 4% and 1% sera, respectively, might be estimated at ~0.46–0.55 and ~0.072–0.084 μg/mL, respectively. Therefore, functional anti-FcεRIα and anti-IgE AAb levels in diluted sera from CSU patients should be detectable by the AlphaCL assay.

The AlphaCL signal counts to detect either anti-FcεRIα or anti-IgE AAbs were obtained in 9 of 12 aiCSU patients who displayed positive results in the ASST (Fig. 4A). The reason why the signal counts for these AAbs could not be observed in sera from three aiCSU patients with a positive ASST is presently unclear. However, two possible hypotheses may explain this contradictory result. First, the sensitivities for the detection of anti-IgE AAbs in our assay might be low. Indeed, our assay could detect anti-IgE AAbs in only two of four aiCSU patients who displayed higher values in the HRT using lactic acid-untreated leukocytes (Table 1, Fig. 4B). In addition,

a 24-h incubation was needed to detect anti-IgE AAbs in our assay (Fig. 3A). As shown in Figure 3B, the elevated signal counts gradually decreased in a concentrations-dependent manner when the serum concentration was >1%, suggesting that the crosslinking of IgE Abs on the surfaces of beads with anti-IgE AAbs might be disturbed by highly abundant serum components, including albumin and free IgE Abs. Therefore, it may be necessary to purify the IgG Abs, or to remove the disturbing components in the serum to achieve higher sensitivity in our anti-IgE AAb assay. Second, several factors, including coagulation factors,^{17,32} substance P,^{33–35} complement^{17,36} and IgE AAbs,⁷ in the patients' sera might induce histamine release from mast cells and basophils in the ASST. Fagiolo *et al.* reported that IgG-depleted sera elicited positive HRT reactions comparable with those observed with untreated sera.³⁷ Further studies are necessary to clarify the positive HRT reactions induced by non-IgG AAbs.

No significant correlation was observed between anti-FcεRIα AAb levels and an FcεRI-crosslinking ability in sera from aiCSU patients (Table 1, Fig. 4A). In this study, anti-FcεRIα AAbs were detected by ELISA even in healthy subjects. Fiebiger *et al.* reported that anti-FcεRIα AAb titers by ELISA were similar between CSU patients and patients with classic skin-related autoimmune diseases, including pemphigus vulgaris, dermatomyositis, systemic lupus erythematosus and bullous pemphigoid, whereas anti-FcεRIα AAbs from CSU patients alone displayed histamine-releasing activity.³⁸ These and other researchers

reported that the anti-FcεRIα AAbs in CSU patients predominantly consisted of the IgG subtypes IgG₁ and/or IgG₃, whereas anti-FcεRIα AAbs in dermatomyositis patients were mainly the IgG₂ and/or IgG₄ subtypes.^{21,38,39} These results suggest that individual proportions of the anti-FcεRIα IgG subtypes may cause differences in individual histamine–release activity among aiCSU patients. In addition to IgG subtypes, immunoglobulin classes other than IgG might be ascribed to the non-correlation between the anti-FcεRIα AAB levels and its FcεRI-crosslinking ability in aiCSU patients. In this study, we detected anti-FcεRIα IgG AAbs as anti-FcεRIα AAbs using ELISA (Table 1). Altrichter *et al.* recently reported that serum anti-FcεRIα IgM levels were increased in CSU patients who displayed a positive ASST result.²⁶ Although they did not confirm whether the anti-FcεRIα IgM AAbs had histamine–release activity in their study, it is possible that anti-FcεRIα IgM AAbs crosslink the FcεRIα proteins on the bead surface and/or interfere with FcεRIα-crosslinking by anti-FcεRIα IgG AAbs. We did not investigate the differences in IgG subtypes or immunoglobulin classes among the sera from the aiCSU patients in the present study. Therefore, further studies are necessary to clarify the differences between the serum anti-FcεRIα AAB levels and FcεRIα-crosslinking ability in terms of IgG subtypes and immunoglobulin classes.

In this study, AlphaCL detected anti-FcεRIα AAbs in four of five patients who displayed negative results for HRT (Fig. 4). The overall limitation in this study was the small number of patients investigated; thus, we could not determine the cut-off value of our assay to diagnose type IIb aiCSU. Therefore, we could not determine whether these signals were truly positive or not. In addition, the degree of IgE- or FcεRIα-mediated basophil activation often varies among basophil donors in HRT results^{15,19}; thus, patients who displayed negative results for HRT may show positive results when we perform the HRT using the other basophil donors. Further studies are necessary to determine its sensitivity, specificity and cut-off value using a large number of CSU patients.

In conclusion, we established a new assay using AlphaCL to detect functional AAbs with FcεRIα- and IgE-crosslinking ability in sera from aiCSU patients. This simple and practical assay method may be available as a diagnostic tool for type IIb aiCSU patients.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

YK, TY, TT, RO and HM conceived and designed the experiments. YK and KI performed the experiments and analyzed the data. YK, TY and HM wrote the manuscript. YC, EM, ST and MH contributed to data collection. EM and MH revised the article critically for important intellectual content. All authors read and approved the final manuscript.

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