



Required cefazolin concentration to maximize diagnostic accuracy of the basophil activation test for cefazolin-induced anaphylaxis

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Abstract

Purpose Identifying the causative agent of perioperative anaphylaxis is key to preventing its recurrence. Besides skin testing, the basophil activation test (BAT) is increasingly being accepted as an additional and reliable method. Cefazolin seems to be a major cause of perioperative anaphylaxis. However, few studies have described use of the BAT for cefazolin-induced anaphylaxis. In this study, we aimed to determine the optimum cefazolin concentration required in the BAT for an accurate diagnosis.

Methods Seven patients who presented with immediate hypersensitivity to cefazolin and 21 control subjects were studied. We conducted skin tests and performed BATs using both CD203c and CD63 as markers of activated basophils. We measured the ratio of activated basophils after stimulation with serial dilutions of cefazolin and investigated the cefazolin concentration that resulted in better sensitivity and specificity.

Results All patients demonstrated positive reactions to cefazolin, while all control subjects showed negative reactions on skin tests. The net percentage of both CD203c- and CD63-labeled activated basophils was greater when higher concentrations of cefazolin than previously reported were used. In control subjects, however, the number of activated basophils by cefazolin stimulation was negligible regardless of its concentration. In the case of CD203c, the sensitivity was 86% with a cefazolin concentration of 3 mg/ml, while in the case of CD63, the sensitivity was 100% with a cefazolin concentration of 10 mg/ml.

Conclusion Using a higher concentration of cefazolin than previously reported for the BAT might increase the accuracy of diagnosis of cefazolin-induced anaphylaxis.

Keywords Anaphylaxis · Cefazolin · Basophil activation test · Skin test

Introduction

In daily clinical practice, antibiotics that are used to prevent surgical site infection are reportedly a major cause of IgE-mediated hypersensitivity reactions during the perioperative period [1–5]. Among them, cefazolin is widely used as the first choice for antimicrobial prophylaxis for many types of clean operations according to the recommendations of several guidelines [6, 7]. In fact, cefazolin is likely to be the most used antibiotic during the perioperative period in many countries, including Japan. Although the incidence of anaphylaxis caused by cefazolin is unknown, it seems to be a major cause of perioperative anaphylaxis, possibly because of its high consumption [4].

Since anaphylaxis is a life-threatening condition with serious consequences, its correct diagnosis is necessary to prevent recurrence [8]. Various diagnostic approaches,

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including medical history taking, skin tests, and measurement of specific IgE levels, are performed to identify the causative agent of anaphylaxis [9]. Nevertheless, there are several problems with these approaches. For example, skin testing carries the risk of serious complications, including recurrence of anaphylaxis and the accompanying painful sensation for subjects [10–14]. Especially in children and pregnant woman, performing skin tests is challenging in most cases. Since a cefazolin-specific IgE measurement kit is not commercially available, development of a novel in vitro approach to diagnose cefazolin-induced anaphylaxis is desired [15].

Recently, the BAT has become accepted as an additional and reliable diagnostic tool to identify the causative agent of immediate drug hypersensitivity with high sensitivity and specificity [15–23]. The utility of the BAT for diagnosing antibiotic-induced anaphylaxis, including by cefazolin, has been reported [15, 21, 24]. A previous study suggested that the appropriate cefazolin concentration for diagnosis with high sensitivity (75%) and specificity (94%) is approximately 0.5 mg/ml [15]. Indeed, the ratio of activated basophils often increases by increasing the concentration of the offending drug [17–19]. We hypothesized that the ratio of activated basophils obtained from patients with cefazolin hypersensitivity could increase using a greater cefazolin concentration, which in turn might result in greater sensitivity and specificity of the BAT. To test this hypothesis, we performed BATs in patients who showed positive reactions to skin tests and control subjects to determine the optimal concentration of cefazolin for the BAT.

Materials and methods

Subjects

This prospective observational study conforms to the standards of the Declaration of Helsinki and was approved by the Institutional Review Board of Gunma University Hospital (ID: 1084, 1259, 150034). The study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR ID: 000015751, 000016761, and 000022365). This manuscript adheres to the applicable STROBE guidelines. Patients and controls were selected as described in our previous study [19]. Briefly, we recruited seven patients who presented with immediate hypersensitivity to cefazolin during anesthesia between July 2014 and December 2017. To confirm the specificity of the BAT, we recruited another group of 21 healthy volunteers as controls. A healthy volunteer was defined as one without allergic diseases, pregnancy or any remarkable medical history. The control subjects in the current study were exactly the same as those who participated in our previous study [19]. All

patients and/or their guardians and controls provided written informed consent for study participation.

Skin and blood tests

Skin testing with cefazolin was performed according to several international guidelines [1, 25–27], and a maximum cefazolin concentration of 20 mg/ml was applied [8]. The aim of the skin tests was to diagnose cefazolin-induced anaphylaxis in patients. Skin prick test (SPT) reactions were considered positive when the size of the wheal increased by 3 mm or more in diameter after 20 min and was associated with a flare [1]. Intradermal tests (IDTs) were performed to create a post-injection wheal of up to 4 mm in diameter. IDT reactions were deemed positive when the diameter of the reactionary erythematous wheal was equal to at least twice that of the post-injection wheal after 20 min [1]. In all cases, IDTs were preceded by SPTs. When the SPTs showed positive reactions, we did not perform IDTs. In all cases with negative reactions to SPTs, we performed IDTs.

Optional blood tests for plasma histamine and serum tryptase concentrations were performed in five cases. The thresholds to determine the elevation of histamine and tryptase concentrations were set at 1 ng/ml and 11.4 µg/l, respectively. Blood samples were collected at the earliest opportunity after the start of the reactions in the operating room.

Basophil activation tests

The methods of the BAT are detailed elsewhere [19, 28]. Briefly, we obtained whole blood samples from patients with positive reactions to cefazolin in skin tests. The BAT was performed using a flow cytometer (FACS Canto II, BD Biosciences, San Jose, CA, USA). For detecting activated basophils, an Allergenicity kit (Beckman Coulter Inc., Brea, CA, USA) was used within 4 h after blood sampling. Blood samples were incubated with serial dilutions of cefazolin (0.006, 0.06, 0.6, 3, and 10 mg/ml) at 37 °C. Anti-IgE-antibody and phosphate buffered saline (PBS) were used as positive and negative controls, respectively. The samples were stained with 20 µl of a mixture containing anti-CRTH2-FITC, anti-CD203c-PE, and anti-CD3-PC7-conjugated antibodies. Basophils were selected based on a side scatter and low CD3[−]/CRTH2⁺ gate profile and at least 500 basophils were counted. Furthermore, we counted the number of basophils with CD63 in another set of samples. For this set of samples, anti-CD63-PE-conjugated antibodies (Beckman Coulter Inc., Brea, CA, USA) were used instead of anti-CD203c-PE-conjugated antibodies.

The results are expressed as the net percentage of activated basophils, calculated by subtracting spontaneous CD203c expression (negative control) from the value

obtained with cefazolin stimulation [18]. A maximum of 5% spontaneous expression of CD203c after stimulation with the buffer solution (negative control) was tolerated. The net percentage of activated basophils was calculated using the following formula: number of activated basophils/total number of basophils $\times$ 100 (i.e., the percentage of activated basophils) – percentage of spontaneous CD203c expression. The same calculation method was applied for CD63 counting.

Statistical analysis

To compare the extent of basophil activation between patients and control subjects, the area under the concentration response curve for BATs and its 95% confidence interval (CI) were calculated in each group, and the results were compared using the Mann–Whitney *U* test. The median values of the net percentage of activated basophils were used for calculating areas under the curves (AUCs). Hodges–Lehmann estimator and its 95% CI were also calculated for assessing the median difference in the AUCs between patient and control groups. Two-graph receiver operating characteristic (TG-ROC) curve analysis was performed to estimate the optimal cut-off value of cefazolin concentration [17, 18]. The number of subjects who showed a greater and smaller percentage of activated basophils than the threshold was compared between patients and control individuals using the Chi square test.

To determine the optimum cefazolin concentration for the diagnosis of cefazolin-induced anaphylaxis in the BAT, the net percentages of activated basophils with cefazolin concentrations of both 3 and 10 mg/ml in patients were compared with those with a cefazolin concentration of 0.6 mg/ml using one-way repeated measures analysis of variance. Dunnett's methods were used for post hoc tests.

To calculate the sample size in this study, we referred to a study on the diagnosis of cefazolin-induced anaphylaxis by the BAT, in which the average percentage of CD203c+ basophils after stimulation with 1100 μ mol/l cefazolin, the equivalent of 0.6 mg/ml cefazolin, was approximately 28% (standard deviation [SD] 8%) in the patient group [15]. Assuming that the average percentage of CD203c+ basophils after stimulation with 10 mg/ml cefazolin could be 38% (SD 8%), a sample size of 6 was required to achieve 80% power to detect a difference between the ratio of CD203c+ basophils after stimulation with 1100 μ mol/l and 10 mg/ml cefazolin with a significance level of $P < 0.05$. Based on these estimates, we included 7 patients and 21 controls in this study.

We used SAS University Edition (SAS Institute Inc., Cary, NC, USA) for calculating Hodges–Lehmann estimator. Power analysis was performed with R ver. 3.3.1. We used Sigma Plot 12.5 (Systat Software Inc., San Jose, CA, USA)

for other analyses. Differences were considered significant at a P value < 0.05 .

Results

All seven patients fulfilled the clinical criteria of anaphylaxis, namely, they showed symptoms related to two or more of the following organ systems soon after exposure to cefazolin: cardiovascular, cutaneous, and respiratory systems (Table 1). In fact, the onsets of these symptoms in all patients were within 20 min after cefazolin administration, suggesting immediate hypersensitivity reactions. In all patients, cefazolin was administered during the period between the induction of anesthesia and commencement of surgery. Although the timing of blood sampling in the operating room varied with each case (10–90 min after the start of reactions), both histamine and tryptase levels were increased in all five patients tested (Table 1). These data also supported a diagnosis of anaphylaxis. None of the patients had a history of allergy to any drugs other than cefazolin. The volunteers, as controls, included 11 females and 10 males, and their ages ranged from 22 to 48 years (median age 26 years).

In the patient group, skin tests were performed 4–18 weeks after the episode (Table 1). In both SPTs and IDTs, none of the control individuals reacted to any of the concentrations of cefazolin tested. In contrast, two patients showed positive reactions in SPTs. In addition, five patients showed positive reactions in IDTs (Table 1). Taken together, all patients showed positive reactions to cefazolin in either SPTs or IDTs, suggesting that cefazolin was indeed the most likely causative agent of anaphylaxis in all the cases.

We confirmed the baseline reactivity of basophils in the patients and control subjects, namely the percentage of basophils that responded to both positive and negative controls (Table 2). To clarify, the percentage of basophils that responded to the positive control was shown without subtracting the percentage of basophils that responded to the negative control (Table 2), while the percentage of basophils that were positive for cefazolin was expressed as the net percentage of activated basophils and was obtained by subtracting the percentage of basophils with spontaneous positivity (see Methods). There was no difference between the two groups in the percentage of CD203c+ basophils for positive and negative controls (Mann–Whitney *U* test, positive control: $P = 0.07$, negative control: $P = 0.44$). This was also true for CD63+ basophils (Mann–Whitney *U* test, positive control: $P = 0.54$, negative control: $P = 0.92$). The number of activated basophils after stimulation with cefazolin was compared by drawing dose–response curves for the patients and control subjects. Overall, the patients, but not controls, demonstrated CD203c up-regulation in a dose-dependent

Table 1 Data of the patients

Patient no.	Age (years)	Sex	Symptoms	Severity grade	Histamine (ng/ml)		Tryptase ($\mu\text{g/l}$)		Delay in skin tests (days) ^a	SPT (mg/ml)	IDT (mg/ml)	Delay in the BAT (months) ^b
					After episode	Baseline	After episode	Baseline				
1	71	F	Cu, Ca	III	11	0.78	13.2	2.9	61	Negative	0.02	2
2	26	F	Cu, Ca	III	115	1	17.7	2.5	55	Negative	0.2	2
3	77	M	Cu, Ca, Re	III	16.8	–	52.5	4	126	1	ND	12
4	67	F	Cu, Ca	II	9.6	–	2.4	–	48	Negative	0.02	2
5	51	M	Cu, Ca	III	–	–	–	–	27	10	ND	4
6	33	F	Cu, Ca	III	49.1	0.1	5	3.6	46	Negative	0.2	5
7	14	M	Cu, Ca	III	–	–	–	–	82	Negative	0.2	3

Demographic characteristics, clinical symptoms, and the results of blood and skin tests in the seven patients included in this study. All patients showed two or more of the following: Ca, Cu, Re, and Ga symptoms. Numerical values indicate the concentration of cefazolin with positive skin reactions

BAT basophil activation tests, Ca cardiovascular, Cu cutaneous, F female, Ga gastrointestinal, IDT intradermal tests, M male, ND tests not performed (negative, tests were performed but proved negative), Re respiratory, SPT skin prick tests

^aInterval between anaphylaxis and investigations, expressed in days

^bInterval between anaphylaxis and investigations, expressed in months. The severity of each case was evaluated according to the criteria proposed by Ring and Messmer [29]

Table 2 Net percentage of activated basophils (median (%) [95% CI])

Marker	Cefazolin (mg/ml)	Patients (%)	Controls (%)
CD203c	PC	79.2 [65.5 to 87.4]	69.8 [23.9 to 93.1]
	NC	5.0 [4.6 to 5.0]	5.0 [4.8 to 5.0]
	10	51.8 [2.8 to 63.6]	–0.5 [–3.7 to 3.9]
	3	36.8 [2.3 to 52.6]	0.1 [–3.6 to 4.3]
	0.6	4.9 [0.3 to 21.9]	0 [–3.2 to 3.4]
	0.06	–0.1 [–1.5 to 2.0]	–0.3 [–3.2 to 3.5]
	0.006	0.3 [–2.8 to 1.1]	0.3 [–3.7 to 8.8]
CD63	PC	53.2 [45.5 to 88.7]	48.6 [26.0 to 81.5]
	NC	5.0 [4.9 to 5.0]	5.0 [4.8 to 5.0]
	10	19.0 [3.5 to 56.0]	–1.8 [–3.4 to 2.6]
	3	11.4 [1.8 to 47.4]	–0.8 [–3.0 to 2.9]
	0.6	2.7 [–2.5 to 7.8]	–0.8 [–4.3 to 5.2]
	0.06	–0.2 [–2.6 to 9.0]	–0.5 [–3.6 to 2.5]
	0.006	0.5 [–2.1 to 3.5]	–1.0 [–3.7 to 0.6]

Percentage of activated basophils after stimulation with positive and negative controls and with serial dilutions of cefazolin. The numerical values in parentheses represent 95% confidence intervals of the median values

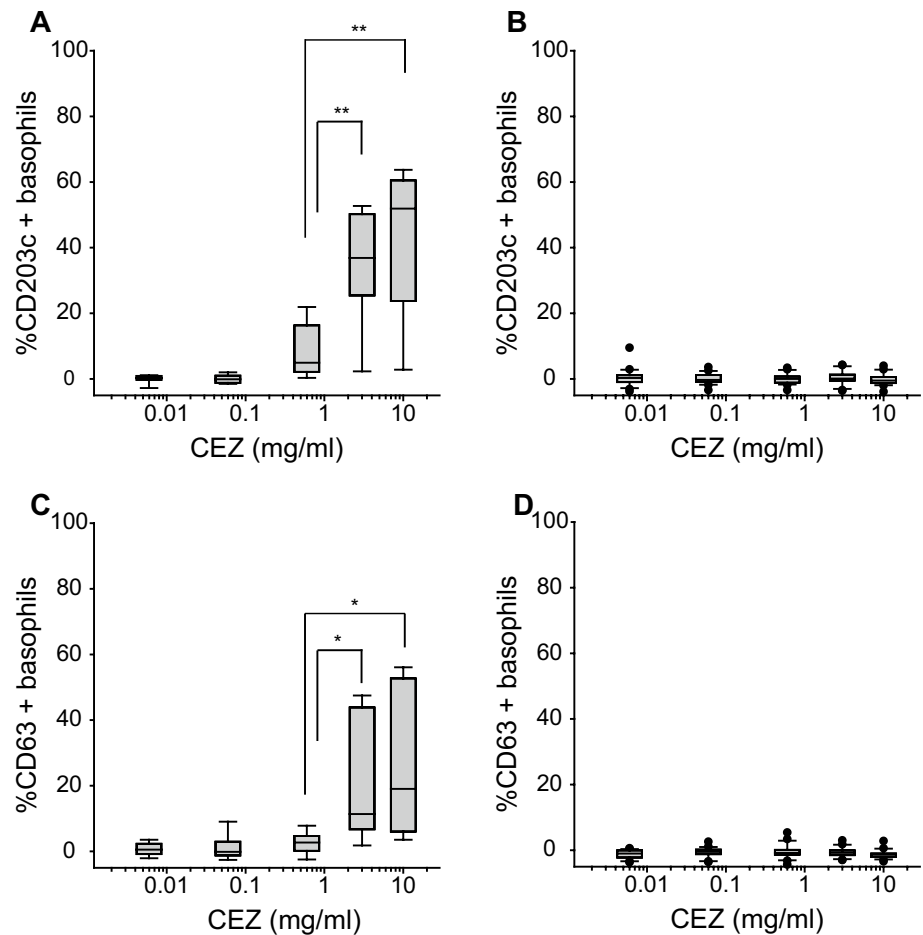
NC negative control, PC positive control

manner (Table 2; Fig. 1a, b). This was also true for CD63 (Table 2; Fig. 1c, d). Since we tested 10 mg/ml cefazolin in only six patients, we calculated the AUCs using the net percentage of activated basophils by stimulation with cefazolin at values ranging from 0.006 to 3 mg/ml. The median values of AUCs in the patients and controls for CD203c were 62.40 (Fig. 1a, 95% CI 10.92–76.67) and –0.36 (Fig. 1b, 95% CI –10.47 to 10.11), respectively (Mann–Whitney *U* test, $P < 0.001$), whereas the AUCs in the patients and controls for CD63 were 17.44 (Fig. 1c, 95% CI –1.526 to 62.53) and –1.87 (Fig. 1d, 95% CI –8.66 to 10.00), respectively (Mann–Whitney *U* test, $P = 0.002$). Hodges–Lehmann estimators of the median differences between patients and controls for CD203c and CD63 were 61.4 (95% CI 53–67.6) and 20.0 (95% CI 12–63.4), respectively. Since the 95% CI of the difference between patients and control groups for both markers did not cross zero, we concluded that there was a statistically significant difference in AUCs between the patient and control groups.

The net percentage of activated basophils labeled with CD203c was greater with both 3 and 10 mg/ml cefazolin compared to 0.6 mg/ml cefazolin (10 mg/ml: $P = 0.002$, 3 mg/ml: $P = 0.006$). Similarly, those with CD63 was greater with 3 and 10 mg/ml cefazolin compared to 0.6 mg/ml cefazolin (10 mg/ml: $P = 0.02$, 3 mg/ml: $P = 0.03$) (Fig. 1).

We performed TG-ROC curve analyses to determine the optimal cut-off values for each concentration of cefazolin corresponding to the best sensitivity and specificity for CD203c and CD63. We determined the optimal cut-off

Fig. 1 Cefazolin-induced CD203c and CD63 up-regulation in patients who presented with immediate hypersensitivity to cefazolin [$n=7$; CD203c (a), CD63 (c)]. The results of BATs in control subjects who demonstrated negative skin reactions to cefazolin are also shown [$n=21$; CD203c (b), CD63 (d)]. The results are expressed as the net percentage of activated basophils. The ends of the boxes define the 25th and 75th percentiles, with the line in the middle representing the median and error bars defining the 10th and 90th percentiles. Outliers are indicated as filled circles in figure parts B and D. Serial dilutions of cefazolin (0.006, 0.06, 0.6, 3, and 10 mg/ml) were used. * $P<0.05$, ** $P<0.005$, one-way repeated measures ANOVA with Dunnett's post hoc test



values, defined as those with maximum specificity (i.e., 100%, if possible) together with the highest sensitivity, for cefazolin concentrations of 0.06, 0.6, 3 and 10 mg/ml (Table 3; Figs. 2, 3). In the case of CD203c, the sensitivity

of the BAT was greatest at a cefazolin concentration of 3 mg/ml (86%, 95% CI, 42–100) (Table 3). In the case of CD63, sensitivity was greatest at cefazolin concentration of 10 mg/ml (100%, 95% CI 48–100) (Table 3).

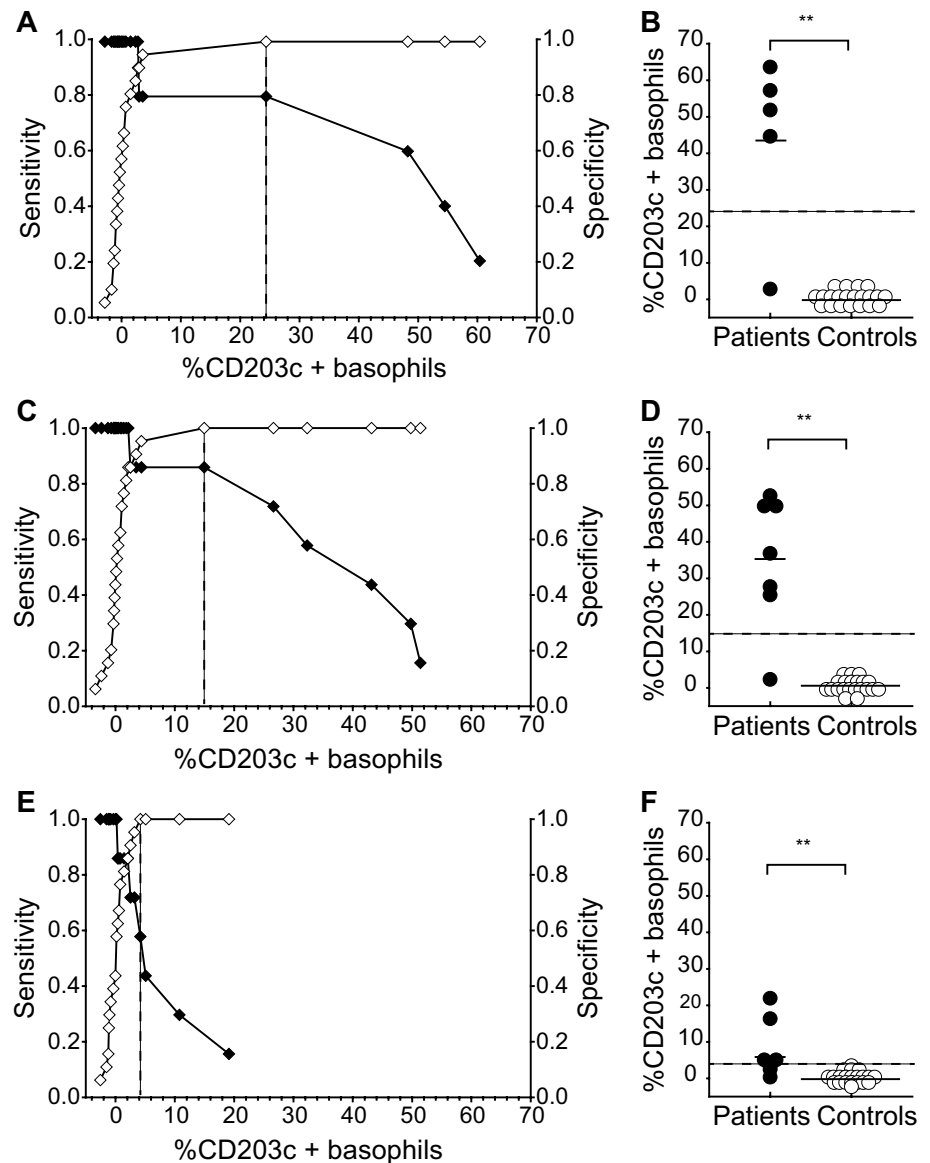
Table 3 Data of ROC curve analyses of the BAT with CD203c and CD63

Marker	Cefazolin (mg/ml)	AUC	Cut-off value (%)	Sensitivity	Specificity
CD203c	10	0.98 (0.94–1.0)	24.3	0.80 (0.28–0.99)	1.0 (0.84–1.0)
	3	0.98 (0.93–1.0)	14.9	0.86 (0.42–1.0)	1.0 (0.84–1.0)
	0.6	0.92 (0.80–1.0)	4.2	0.57 (0.18–0.90)	1.0 (0.84–1.0)
	0.06	0.50 (0.26–0.75)	3.1	0 (0–0.41)	0.95 (0.76–1.0)
	0.006	0.51 (0.27–0.71)	6.2	0 (0–0.41)	0.95 (0.76–1.0)
CD63	10	1.0 (1.0–1.0)	3.2	1.0 (0.48–1.0)	1.0 (0.84–1.0)
	3	0.99 (0.96–1.0)	5.7	0.83 (0.36–1.0)	1.0 (0.84–1.0)
	0.6	0.79 (0.51–1.0)	6.6	0.17 (0–0.64)	1.0 (0.84–1.0)
	0.06	0.60 (0.32–0.88)	5.8	0.17 (0–0.64)	1.0 (0.84–1.0)
	0.006	0.78 (0.53–1.0)	0.9	0.5 (0.12–0.88)	1.0 (0.84–1.0)

Results of receiver operating curve (ROC) analyses for the BAT with CD203c and CD63. The numerical values in parentheses represent 95% confidence intervals

AUC area under the curve, BAT basophil activation tests, ROC receiver operating characteristic

Fig. 2 Two-graph ROC curves of cefazolin-induced CD203c expression generated in cefazolin-allergic patients ($n=7$) and control individuals ($n=21$) (**a** 10 mg/ml, **c** 3 mg/ml, **e** 0.6 mg/ml). The dashed lines denote the threshold for positivity (**a** 24.3%, **c** 14.9%, **e** 4.2%). Open and closed diamonds indicate sensitivity and specificity, respectively. Cefazolin-induced CD203c expression in the basophils of cefazolin-allergic patients ($n=5$ in **A**, $n=7$ in **c** and **e**, closed circles) and control individuals ($n=21$, open circles) (**b** 10 mg/ml, **d** 3 mg/ml, **f** 0.6 mg/ml). The bold and dashed lines denote the medians and threshold, respectively. $**P < 0.005$, χ^2 test



Discussion

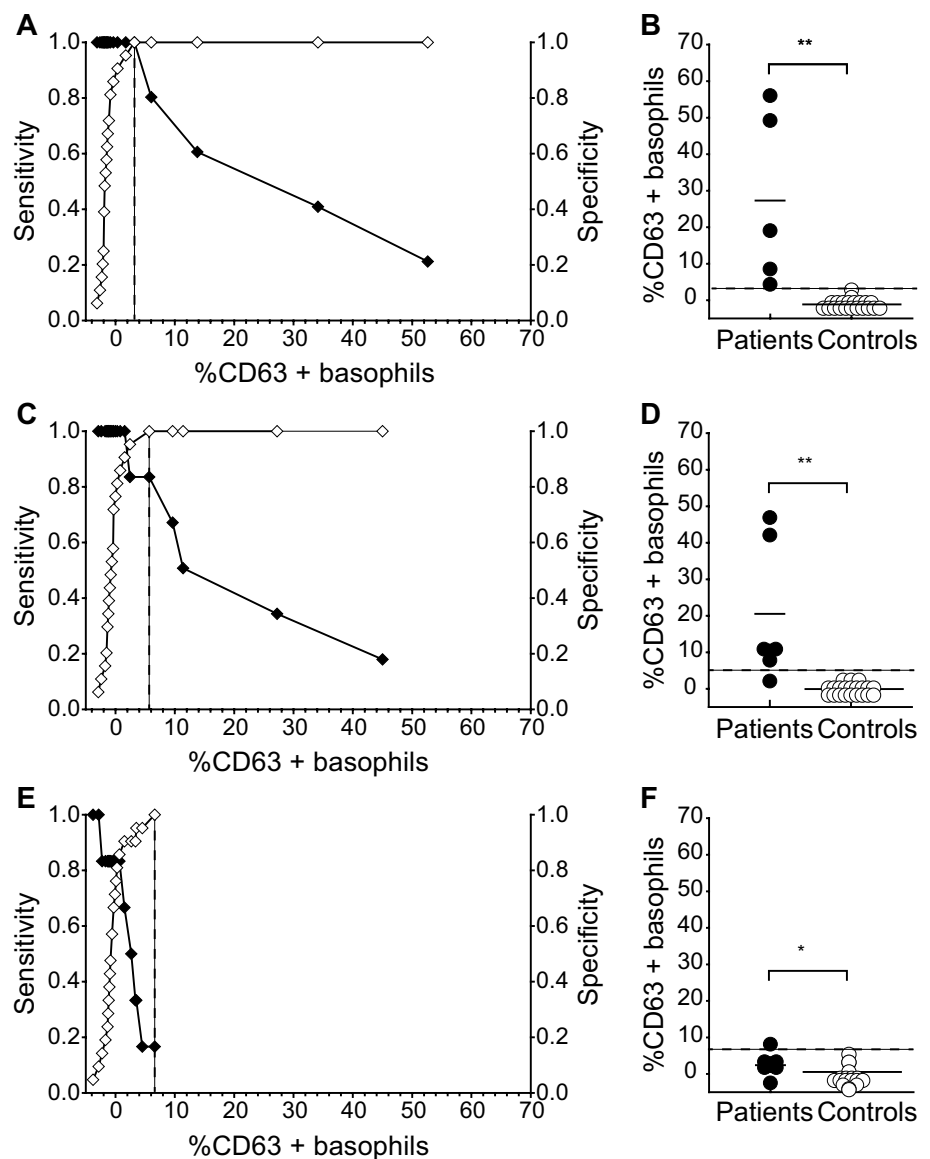
In this study, the patients, but not controls, demonstrated dose-dependent CD203c and CD63 up-regulation following stimulation with cefazolin. The ratio of activated basophils in patients increased with an increasing cefazolin concentration, whereas that of controls remained low. With both CD203c and CD63, sensitivity of the BAT was greatest when higher concentrations of cefazolin than previously reported were used. These results suggest that increasing the cefazolin concentration in the BAT up to 10 mg/ml enhances the diagnostic sensitivity of the test.

As with other drugs, the diagnostic approach to suspected antibiotic-induced anaphylaxis generally relies on skin testing and quantification of the specific IgE. However, novel approaches, such as the BAT, are recently gaining

importance [30]. Compared with quantification of the specific IgE, the BAT shows a higher sensitivity (approximately 50%) and specificity (~90%) [31, 32]. A majority of the studies on the flow cytometric diagnosis of anaphylaxis to antibiotics evaluated amoxicillin [31], there being only a few reports on cefazolin-induced anaphylaxis [15]. In this study, the BAT showed high sensitivity (86–100%) and specificity (100%). These values are even higher than those in previous studies, indicating the usefulness of the BAT for diagnosing cefazolin hypersensitivity [15]. The results of previous studies and the present study suggest that the BAT could contribute to the accurate diagnosis of cefazolin-induced anaphylaxis.

Perioperative use of antibiotics may vary from country to country. Indeed, a recent study conducted in the United Kingdom (UK) showed that the three most commonly

Fig. 3 Two-graph ROC curves of cefazolin-induced CD63 expression generated in cefazolin-allergic patients ($n=6$) and control individuals ($n=21$) (**a** 10 mg/ml, **c** 3 mg/ml, **e** 0.6 mg/ml). The dashed lines denote the threshold for positivity (**a** 3.2%, **c** 5.7%, **e** 6.6%). Open and closed diamonds indicate sensitivity and specificity, respectively. Cefazolin-induced CD63 expression in basophils in cefazolin-allergic patients ($n=5$ in **a**, $n=6$ in **c** and **e**, closed circles) and control individuals ($n=21$, open circles) (**b** 10 mg/ml, **d** 3 mg/ml, **f** 0.6 mg/ml). The bold and dashed lines denote the medians and threshold, respectively. * $P < 0.05$, ** $P < 0.005$, χ^2 test



used antibiotics are gentamicin, which was administered in 20% of all cases, co-amoxiclav (administered in 17%), and cefuroxime (administered in 14%) [33]. However, although there is only limited data on antibiotics used in the perioperative period from countries other than the UK, cefazolin seems to be the most common cause of perioperative antibiotic-induced anaphylaxis in countries other than the UK, because the guidelines for perioperative antibiotics in many countries, including the United States and Japan, recommend cefazolin as the first-choice prophylactic drug for surgical site infection after many types of surgeries [6, 7]. Hence, the outcomes of this research would be of interest to anesthesiologists and allergists in countries using cefazolin abundantly.

Our study has certain limitations. Patients who showed negative reactions to skin tests were not included in this

study. Thus, the negative predictive value of the BAT for patients with suspected cefazolin-induced anaphylaxis is still unknown. Although this study satisfied the requirement of the minimum number of patients and volunteers as estimated by power analysis, prospective trials with more subjects might still be necessary to verify the optimum concentration of cefazolin needed to make a correct diagnosis of cefazolin-induced anaphylaxis by the BAT.

In summary, we found that the sensitivity of the BAT was greatest when using higher concentrations of cefazolin (3 mg/ml and 10 mg/ml) than previously reported (~0.6 mg/ml). Using a higher concentration of cefazolin than previously reported might contribute to more accurate diagnosis of cefazolin-induced anaphylaxis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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