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Original Article

Diagnostic utility of the basophil activation test in natto-induced hypersensitivity

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Abbreviations:

BAT	basophil activation test
PGA	poly γ-glutamic acid
UAS	urticaria activity score
UCT	urticaria control test

ABSTRACT

Background: Natto (fermented soybeans)-induced hypersensitivity is characterized by delayed symptom onset that hampers diagnosis. We aimed to clarify the clinical utility of the basophil activation test (BAT) in the diagnosis of natto-induced hypersensitivity.
Methods: Five patients with a history of anaphylaxis and chronic urticaria suspected of natto-induced hypersensitivity and seven with chronic spontaneous urticaria clinically unrelated to natto were enrolled in the patient and control groups, respectively. The BAT was performed with two incubation times, 15 min and 1 h, in combination with various concentrations of natto-mucilage extract.
Results: In controls, CD203c levels in basophils remained low in the 15-min incubation but were significantly increased in the 1-h incubation. In the patient group, in the 15-min condition, basophil CD203c was significantly upregulated by natto mucilage but not by soybean vs controls ($P = 0.001$). Low concentrations of natto mucilage were sufficient to upregulate basophil CD203c in the anaphylaxis cases, but high concentrations were required to induce the same effect in the urticaria cases. Finally, the dose-dependent pattern of the BAT results differed significantly between the anaphylaxis and urticaria cases ($P = 0.006$). Thus, a strong background reaction was observed in the BAT with 1 h incubation; 15 min of incubation was sufficient to identify patients with natto-induced hypersensitivity and may distinguish the clinical phenotype of natto-induced hypersensitivity, i.e., anaphylaxis or urticaria.
Conclusions: The BAT with a 15-min incubation period is useful in diagnosing natto-induced hypersensitivity.
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Introduction

Bacillus subtilis-fermented soybeans products are widely consumed as a local food in Asia and Africa.[1] Natto, one such product, is a traditional Japanese fermented soybeans food.[2] The natto mucilage produced during fermentation gives natto its characteristic flavor and is a component unique from the soybean itself. Natto has recently been reported to cause anaphylaxis[3,4] and urticaria.[5] Natto-induced hypersensitivity is characterized by its late onset, with symptoms occurring 5–12 h after ingestion. This delay in symptom onset makes it difficult to diagnose natto-induced hypersensitivity in patients merely from medical interviews due to the interval between natto ingestion and symptom onset.

Type I allergies are generally diagnosed via allergen-specific IgE detection, skin tests, and oral food challenges. However, while allergen-specific IgE detection and skin tests are highly sensitive, they are only modestly specific, and skin tests and oral food challenges can be burdensome for both patients and physicians.[6] In contrast, the basophil activation test (BAT), an *in vitro* assay, is generally accepted as a safe and reliable diagnostic assay for food allergies.[6] Unlike regular food, the handling of natto mucilage for diagnostic purposes is rare and its unusual stickiness can contribute to methodological differences between studies. Moreover, in addition to the technical issues, the usefulness of BAT and other *in vitro* methods for diagnosing natto-induced hypersensitivity remains limited.[7–9]

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In this study, the practical utility of BAT was investigated using a detailed constant diagnostic method for natto-induced hypersensitivity. Importantly, a technical pitfall that affects natto-mucilage antigenicity was uncovered.

Methods

Patients

This study was performed in five patients with natto-induced hypersensitivity and seven control patients. The patients presented at the Department of Dermatology, Tokyo Dental College Hospital and Keio University Hospital between February 2017 and June 2021.

The five patients were initially diagnosed with anaphylaxis or chronic urticaria and were suspected to have natto-induced hypersensitivity. Consequently, they were enrolled in the natto-induced hypersensitivity group (Table 1). Cases 1 and 2 experienced anaphylaxis, while Cases 3, 4 and 5 developed only urticaria. Case 3's detailed clinical history and examination results were reported previously.[5] The natto-induced hypersensitivity group exhibited obvious allergic symptoms after natto consumption and/or had a positive scratch test for natto mucilage. In all cases, symptomatic improvement was clinically observed once intake of natto and poly γ-glutamic acid (PGA), the main component of natto mucilage used in many foods, was avoided.[10,11] Notably, the patients were negative for soybean-specific IgE and did not develop allergic symptoms on soybeans ingestion.

The control group consisted of patients with chronic urticaria. These patients did not develop any symptoms following natto or soybeans ingestion and had negative results for soybean-specific IgE and in natto-mucilage and soybean skin tests.

This study was approved by the institutional ethics committees of Tokyo Dental College (approval number I 18–52) and Keio University (approval number 20110133). Informed consent was obtained from all participants.

Laboratory tests

Serum levels of total IgE and soybean-specific IgE (ImmunoCAP; Thermo Fisher Scientific, Waltham, USA) were measured.

Table 1
Clinical characteristics and test results of the four patients enrolled in this study.

Patient number	1	2	3	4	5
Age, sex	50, M	52, M	16, F	65, F	38, F
Symptoms	U, Dy, Di	U, Dy	U	U	U
Serum total IgE (IU/mL)	230	650	23	240	72
Interval between natto ingestion and onset (h)	First time: 5 Second time: 8	7	NM	NM	NM
History of jellyfish stings	Yes	Yes	No	No	No
Soybean-specific IgE (IU/mL)	<0.1	<0.1	<0.1	<0.1	<0.1
Scratch tests (wheal/erythema [mm]) at 15 min					
Histamine chloride	7.5/15	NT	8/16	7/21	NT
Saline	0/0	NT	0/0	2/0	NT
Natto mucilage	22.5/25	NT	0/3 at 15 min 5/18 at 3 h	7/13.5	NT
Soybean	0/0	NT	4.5/7.5	5/10.5	NT
Reference	NA	NA	4	NA	NA

U, urticaria; Dy, dyspnea; Di, diarrhea; NM, not measured; NT, not tested; NA, not applicable. Mean wheal diameter ≥3 mm was defined as positive based on the recommendations of the American Academy of Allergy, Asthma, and Immunology and the American College of Allergy, Asthma, and Immunology.[6]

Skin tests

Scratch tests were performed on the flexor surface of the patients' forearms using lancet needles. Fifteen minutes later, the evaluations were performed following the standard methods recommended by the American Academy of Allergy, Asthma, and Immunology and the American College of Allergy, Asthma, and Immunology.[6] The elicited responses were considered positive when the average allergen-induced wheal diameter was 50 % or greater than that induced by histamine dihydrochloride (10 mg/mL), the positive control. Physiological saline was used as the negative control.

The skin tests were performed using commercially available natto (Takanofoods, Ibaragi, Japan) and soybeans (Torii Pharmaceutical, Tokyo, Japan). The natto was gently stirred in a circular motion approximately 30 times, without crushing the beans, until it became sticky. A small amount of the natto mucilage was applied to the skin, which was then scratched with the lancet. All allergens were introduced to the seven control participants.

BAT

The BAT was performed in BML, Inc. (Saitama, Japan) using an Allergenicity Kit (Beckman Coulter, Brea, CA, USA). After incubating whole blood and antigen, basophil activation was assessed using antibodies against CD3, CRTH2, and CD203c. CD203c expression levels were measured in basophils identified in flow cytometry as $CD3^{-}CRTH2^{+}$ cells.

The BATs were performed under two different incubation times (15 min and 1 h). The allergen solution (50 μL), heparinized whole blood (50 μL), and antibodies (15 μL) were incubated at 37 °C for 15 min. In cases where the incubation time was 1 h, the antibodies were added in the last 15 min. The anti-IgE antibody was used as a positive control, and phosphate-buffered saline was used as a negative control. At least 550 basophils were analyzed using a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA).

Natto-mucilage extract, frozen natto-mucilage extract, and soybean extract were used as antigens. The natto-mucilage extract was prepared by gently stirring the natto (Takanofoods, Ibaragi, Japan) approximately 50 times until it became sticky. Afterwards, 30 mL distilled water at room temperature was poured into 30 g of the stirred natto. It was then mixed gently an additional 20 times in a figure-eight motion until smooth, and left to stand for one hour at room temperature. All procedures were performed carefully to create a natto-mucilage solution without crushing the soybeans. Then, the supernatant was filtered using a 0.45-μm Millex-HP syringe filter unit (Merck Millipore, Germany). The concentration of the filtered supernatant was 36.0 ± 5.0 mg/mL (n = 4) measured by NanoDrop 2000c (Thermo Fisher Scientific, Waltham, USA). Serially diluted samples were mixed with patients' whole blood resulting in final dilution factors of 1/2, 1/8, 1/20, 1/80, 1/200, or 1/800, depending on the assay. Filtered supernatant that was stored at −80 °C was used as frozen natto-mucilage extract. Dilution of the frozen extract was performed in the same manner as that for the natto-mucilage extract. As for the soybean extract, 0.3 g of mashed commercially available boiled soybeans (Marusan-Ai, Aichi, Japan) were stirred with 30 mL distilled water. A concentration of 10,000 μg/mL was filtered. Next, serially diluted samples were mixed with patients' whole blood resulting in final concentrations of 1000, 300, 100, 30, 10, and 3 μg/mL, depending on the assay. Antigen-induced basophil activation rate was calculated as: (antigen-induced $CD203c^{+}$ basophils (%) - spontaneous $CD203c^{+}$ basophils (%))/($CD203c^{+}$ basophils with anti-IgE antibody stimulation (%) - spontaneous $CD203c^{+}$ basophils (%)). If (antigen-induced

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CD203c$^+$ basophils (%) - spontaneous CD203c$^+$ basophils (%)) is less than 0, the value was set as 0.

Statistical analysis

Statistical analyses were performed using paired *t* test, Mann–Whitney *U* test and two-way repeated measures ANOVA with GraphPad Prism 9 (San Diego, CA, USA). Two-sided *P*-values of less than 0.05 were considered statistically significant.

Results

Clinical characteristics of natto hypersensitive patients

Five patients ranging from 16 to 65 years old (mean age, 44.2 years) were suspected of having natto-induced hypersensitivity based on their clinical courses and their laboratory or scratch test results (Table 1). Based on their current main symptoms after natto ingestion, the patients were classified into two categories. The anaphylaxis patients (Cases 1 and 2) developed anaphylactic reactions involving more than two symptoms in different organs including the skin, mucosa, or the respiratory, gastrointestinal, or circulatory systems, as defined previously.[12] In contrast, the urticaria patients (Cases 3, 4 and 5) usually developed urticaria over a long period of time.

The anaphylaxis patients (Cases 1 and 2) experienced anaphylaxis once or twice after ingesting natto. The average interval between natto ingestion and symptom onset was 6.6 h. Both patients experienced generalized urticaria and dyspnea, while Case 1 also developed diarrhea. Notably, the stings from jellyfish nematocysts contain PGA and can cause PGA sensitization.[9] Both patients surfed often and had been stung several times by jellyfish. Importantly, the anaphylaxis patients could consume soybeans with no adverse effects. After their consumption of natto and PGA-containing foods was restricted, anaphylaxis no longer reoccurred.

The urticaria patients (Cases 3, 4 and 5) were initially, and for a lengthy period, considered as having chronic urticaria with an unknown etiology. Soybeans intake did not result in urticaria symptoms in all patients, and none of them had experienced jellyfish stings. After their consumption of natto and PGA-containing foods was restricted, their weekly urticaria activity scores (UAS7) and the urticaria control tests (UCTs) improved markedly, with the scores changing from 28 to 0 in the UAS7 and from 3 to 16 in the UCT in Case 3, from 42 to 7 in the UAS7 and from 0 to 12 in the UCT in Case 4 and from 42 to 2 in the UAS7 and from 1 to 14 in the UCT in Case 5, respectively ($P = 0.01$ for UAS7, $P = 0.007$ for UCT, paired *t* test, Supplementary Fig. 1).[13]

Laboratory tests and skin tests

The total IgE concentration was 23–3200 IU/mL (overall mean, 887 IU/mL; patient group mean, 243 IU/mL; control group mean, 1347 IU/mL). The control group displayed significantly higher levels of total serum IgE than the patient group. Soybean-specific IgE was negative in both groups (Table 1). Moreover, the anaphylaxis patients tested negative for specific IgEs against testable ingredients consumed half a day prior to the episodes. The urticaria patients tested negative for specific IgEs against ingredients frequently consumed in their daily lives based on food diaries and medical interviews. All patients were able to eat these food items without any problems.

Scratch tests were performed on all patients and controls except for Case 2 and Case 5 (Table 1). In the scratch tests for natto mucilage, Cases 1 and 4 were positive, while Case 3 was negative 15 min after scratching. However, as previously described, Case 3 developed a more pronounced reaction to the natto mucilage 3 h after the test.[5] This delayed reaction is consistent with the gradually enhancing and long-lasting reactions following skin tests previously reported.[5,14,15] The scratch tests for soybeans were negative in Case 1 and positive in Cases 3 and 4. After eating soybeans, Case 3 experienced only slight itching but did not develop wheals, while Case 4 was able to consume soybeans without any allergic symptoms. Meanwhile, the results of all scratch tests were negative in the control group.

BAT

BATs with a 15-min but not 1-h incubation period detected hypersensitive states in patients with natto-induced hypersensitivity

Various durations have been previously reported for the allergen incubation time in the BAT.[16,17] In this study, 15-min and 1-h incubation periods, in which whole-blood samples were exposed to antigens for 15 min and 1 h, respectively, were investigated. First, the BAT results for natto mucilage in the control group between the 15-min and 1-h incubation periods were compared. The proportion of CD203c$^+$ cells in basophils remained low in the 15-min condition but was significantly increased in the 1-h condition ($P = 0.02$ for 1/20 and 1/2 dilution, Mann–Whitney *U* test, Fig. 1, Supplementary Fig. 2). This result implies that non-specific eosinophil activation in controls occurred after 1 h of incubation. Therefore, 15 min of incubation may be more appropriate for evaluating natto-induced hypersensitivity than 1 h.

After 15 min of incubation with natto mucilage, CD203c was upregulated in the patient group but not in the control group ($P = 0.001$, two-way repeated measures ANOVA, Fig. 2A, Supplementary Fig. 3A, B). Prominent reactions were not observed for soybeans (Fig. 2B). These results indicate that CD203c upregulation in basophils was detected in the BAT following 15 min of incubation with natto mucilage in patients with natto-induced hypersensitivity.

Anaphylaxis and urticaria cases displayed different patterns in dose-dependent responses of BAT following a 15-min incubation with natto mucilage

The dose-dependent responses of the BAT results following a 15-min incubation with natto mucilage showed two distinct patterns

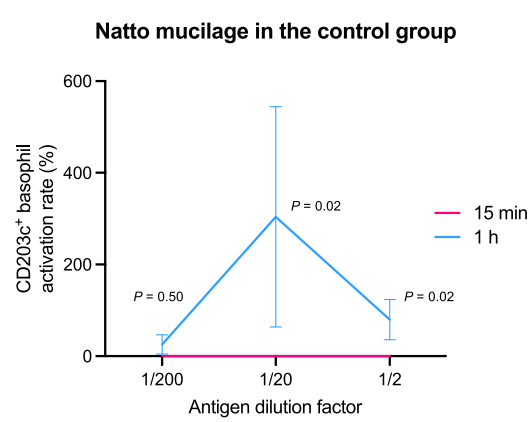

Fig. 1. Upregulation of basophil CD203c in the BAT was detected after 1 h, but not 15 min, of incubation in the control group. BAT results for the control patients under the 15-min (pink line) and 1-h (light blue line) incubation conditions are shown as means ± SEMs. Significant differences were evaluated by Mann–Whitney *U* test for each dilution factor (15 min vs 1 h). See Supplementary Figure 2 for the reference of raw data (% of CD203c$^+$ cells in basophils) and original FACS plots.

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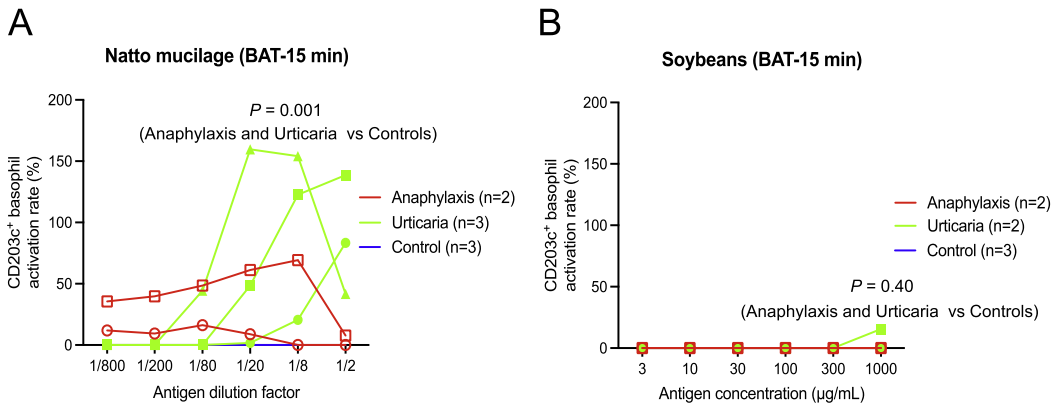

Fig. 2. The results of basophil activation rate in the BAT for natto mucilage and soybeans under the 15-min incubation condition. Dose–response curves for natto mucilage **(A)** and soybeans **(B)** in the 15-min condition are shown in anaphylaxis cases (red line), urticaria cases (green line) and control patients (blue line). Data from control patients are shown as means ± SEMs. Significant differences were evaluated by two-way repeated measures ANOVA (anaphylaxis and urticaria vs controls). See Supplementary Fig. 3A for the reference of raw data and original FACS plots.

that depended on the main clinical phenotype, i.e., anaphylaxis or urticaria, of natto-induced hypersensitivity. CD203c was upregulated at the lowest natto-mucilage concentration (1/800) in the anaphylaxis cases but not in the urticaria cases. The urticaria case required higher concentration of natto mucilage (at least, more than 1/20 dilution) to obtain upregulation of basophil CD203c (Fig. 2A). Furthermore, basophil activation rates were standardized by setting basophil activation rates at the lowest antigen concentration as 100 % to readily see the difference in the dose-dependent response pattern among tested cases (Supplementary Fig. 4). Indeed, the dose-dependent response patterns were significantly different between the anaphylaxis and urticaria cases ($P = 0.006$, two-way repeated measures ANOVA). These results imply that the clinical-phenotype-dependent patterns in the BAT results for natto-induced hypersensitivity may be useful for clinical diagnoses.

Antigenicity of natto mucilage was lost by freeze-and-thaw

To perform the BAT against natto mucilage under constant conditions, it was considered useful to determine whether the natto-mucilage extract can be frozen and stocked and used as antigens of the same lot. Therefore, the antigenic properties of freshly prepared and frozen natto mucilage were compared with respect to their ability to induce CD203c upregulation in basophils in the BAT. In the anaphylaxis group, dose-dependent response patterns of BAT results were almost identical between the freshly prepared and frozen natto mucilage (Fig. 3A). However, in the urticaria group, CD203c upregulation was significantly reduced when frozen natto mucilage was used as antigen compared with freshly prepared natto mucilage ($P = 0.001$, two-way repeated measures ANOVA, Fig. 3B). These results indicate that natto-mucilage antigenicity is affected by freeze-and-thaw procedures and that frozen-stocked natto mucilage may be inappropriate to use in diagnosing natto-induced urticaria.

Discussion

In this study, the BAT for natto mucilage under the 15-min incubation condition was demonstrated to be effective in detecting natto-induced hypersensitivity in patients *in vitro*. Depending on the clinical phenotype of natto-induced hypersensitivity, i.e., anaphylaxis or urticaria, the BAT results after 15 min of incubation with natto mucilage displayed two distinctive dose-dependent patterns. Additionally, the antigenicity of the natto mucilage was affected by the freeze-and-thaw process, indicating that freshly prepared natto mucilage is more appropriate for diagnosing natto-induced hypersensitivity, especially for natto-induced urticaria.

In this study, five cases of natto-induced hypersensitivity were enrolled. Case 1 was a typical case of natto-induced, late-onset anaphylaxis with positive scratch test results. In Case 2, the skin test was not performed because the patient had relocated. However, she had a convincing history of anaphylaxis developing 7 h after ingesting natto. Moreover, she was able to consume other food items, including soybeans, that she had consumed half a day prior to symptom onset, and she had negative results in IgEs specific to them. Also, the patient had a history of jellyfish stings, supporting possible sensitization to PGA. After restriction of only natto and PGA-containing foods, the symptoms did not recur. Together with the typical clinical course of natto allergy and routine blood tests, she was highly suspected of natto-induced anaphylaxis. The positive BAT results obtained in this study was very consistent with the clinical information and was useful for making the diagnosis in this case. In Case 3[5], the urticaria developed in the past decade without any extracutaneous symptoms. An anaphylactic reaction did occur after ingesting a meal including natto once about 2 years ago. But it is doubtful whether natto induced anaphylaxis or not, because there has been no recurrence of anaphylaxis since then despite frequent natto intake. In this patient, however, restriction of only natto and PGA-containing foods dramatically improved her urticaria. Therefore, together with the long-lasting skin reaction in the scratch test[14,15] and the positive BAT results, Case 3 was, at least, considered to have urticaria by natto. In Case 4, the scratch test for natto was positive, and natto restriction markedly improved her urticaria. Therefore, Case 4 was diagnosed with natto-induced urticaria. Together, these four cases imply that even if natto-induced hypersensitivity is clinically suspected, it may be difficult to obtain typical findings from conventional skin tests. However, the BAT can overcome the diagnostic difficulties in natto-induced hypersensitivity. In Case 5, she had suffered from urticaria every day. Her symptom develops throughout the day, since starting to eat natto for her health three years ago. Soybeans and other food stuffs usually consumed did not induce urticaria, and soybean-specific IgE was also negative. After restricting the intake of natto and other food items containing PGA, her urticaria almost disappeared. Although we were unable to perform the skin test, we suspected

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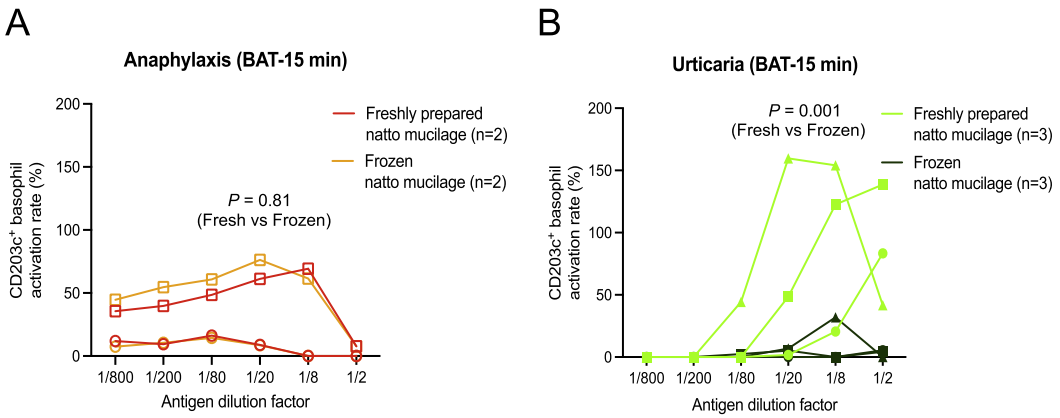

Fig. 3. Basophil activation from frozen natto mucilage versus freshly prepared antigen was reduced in urticaria cases but not in anaphylaxis cases. **(A, B)** Dose–response curves to freshly prepared (red and green lines) and frozen (yellow and dark green lines) natto mucilage in the 15-min condition are shown in anaphylaxis **(A)** and urticaria cases **(B)**. The data for the freshly prepared antigen shown in Figure 2A were re-used. Each mark (triangles, squares and circles) represents each case. Significant differences were evaluated by two-way repeated measures ANOVA (fresh vs frozen natto mucilage).

that her symptoms were caused by natto based on her clinical course and the results of BAT.

Generally, IgE-mediated food allergies are diagnosed based on clinical history and assays such as specific IgE detection, skin prick test, and oral challenge test. However, the skin and oral challenge tests risk the development of allergic reactions, and the specific IgE detection do not distinguish between allergic and tolerant patients. In natto-induced hypersensitivity, typical results from skin tests are sometimes difficult to obtain compared to common food allergies. Therefore, other diagnostic tools are required to assist in patient diagnosis and management. Regarding *in vitro* tests for detecting natto-induced hypersensitivity, an ELISA usually detects IgE with high sensitivity, but it is still under development.[8] The BAT has shown positive results against jellyfish and PGA in patients with natto-induced anaphylaxis,[7,9] suggesting that the BAT is a possible tool for efficiently diagnosing natto-induced hypersensitivity as a simple, low-risk test method that requires only small blood samples that could be performed prior to the skin tests. Indeed, in this study, the BAT applied to natto mucilage under a 15-min incubation period was demonstrated to discriminate between the patients and the controls.

One of the other advantages of the BAT is the flexibility of testable antigens. The BAT can essentially examine any antigen of interest.[18] However, this aspect is also a disadvantage since it is necessary to establish a standard method for each antigen preparation. This requirement is difficult to meet when the antigen is rare. Here, an appropriate method to prepare natto-mucilage extract of sufficiently high quality to obtain proper BAT results was described; its stickiness makes it difficult to handle when making an extract for use as an antigen.

Regarding incubation times, a 15-min incubation period is generally considered sufficient for the BAT,[19] but some antigens have been incubated for 1 h or even longer.[16,19] The basophil activation markers, CD203c and CD63, were upregulated and reached a steady state after 1 h of incubation.[16] In previous cases of natto-induced hypersensitivity, the BAT for jellyfish and PGA was positive after 1 h.[7,9] However, in this study, upregulation of CD203c in basophils after exposure to natto mucilage was observed in both the patient and control groups after 1 h of incubation. A variety of metabolites produced in the natto mucilage during fermentation likely induced the non-specific basophil activation in the 1-h condition. However, the non-specific reaction was not observed in a shorter incubation time, which in this study was at least 15 min, thereby highlighting the antigen-specific basophil activation. Accordingly, the BAT for natto mucilage in the 15-min incubation condition is an appropriate method to detect natto-induced hypersensitivity.

Notably, the responses in the BAT differed between patients with anaphylaxis versus those with urticaria. The anaphylaxis cases responded to lower concentrations of natto mucilage compared to the urticaria cases. This result is consistent with previous studies reporting that patients with severe reactions to allergens tend to have higher basophil sensitivity in the BAT, with patients reacting to lower doses being more at risk of severe symptoms.[20] In addition, ideal BAT results were obtained in the anaphylaxis cases, which showed a bell-shaped curve.[21] In contrast, although the BAT results in the urticaria cases except Case 5 were not ideal, basophil activation was detected in a high concentration of natto mucilage, suggesting the BAT can, at least, assist in diagnosing natto-induced urticaria. Compared to the anaphylaxis cases, natto-mucilage-specific IgE on basophil surfaces in the urticaria may be less abundant or have lower affinity.[21,22] Taken together, the 15-min BAT results may be associated with patients' clinical symptoms, and over multiple antigen concentrations, they may cover the spectrum of immediate-type natto hypersensitivity ranging from urticaria to anaphylaxis. Patients who react to lower doses might be more at risk of severe symptoms even in natto-induced hypersensitivity, as previously suggested.

The structure and properties of PGA can change depending on the environmental conditions, such as pH, polymer concentration, and ionic strength.[10,23] Temperature is no exception. As shown in Figure 3, CD203c upregulation was similar to the levels observed in the negative controls when frozen natto mucilage was tested in the urticaria group. Therefore, the freeze-and-thaw procedure may affect natto-mucilage antigenicity and reduce the detection sensitivity of the reaction, especially in patients with natto-induced urticaria. Unfortunately, frozen natto is exported overseas from Japan. Accordingly, it may be difficult to prepare the appropriate natto antigens from fresh natto for the BAT outside Japan, although frozen natto remains useful for diagnosing anaphylaxis. The future use of PGA as an allergic component is one option to overcome this technical hurdle. The suitable chemical form of PGA for BAT needs to be characterized in further investigations.

Natto-induced hypersensitivity is not a local clinical issue, such as only in Japan. PGA sensitization can be caused via stings by jellyfish nematocysts, which contain PGA, as well as via oral ingestion of natto.[9,14] Therefore, people who engage in marine activities tend to have a higher risk of PGA sensitization due to exposure to jellyfish stings. Additionally, the consumption of natto has increased

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worldwide due to its reputation as a health food. Moreover, PGA is contained in many Asian fermented soybeans products[11] and is also widely used industrially in food, beverages, cosmetics, and medicine.[10] Therefore, patients with natto-induced hypersensitivity may exist worldwide. In this study, the utility of the BAT to diagnose patients with natto-induced hypersensitivity, including both anaphylaxis and urticaria, was demonstrated. Although the number of patient samples was limited due to their rarity, the data from this study may help improve the identification and management of patients that physicians in other countries may encounter.

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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.2021.07.010.

Conflict of interest

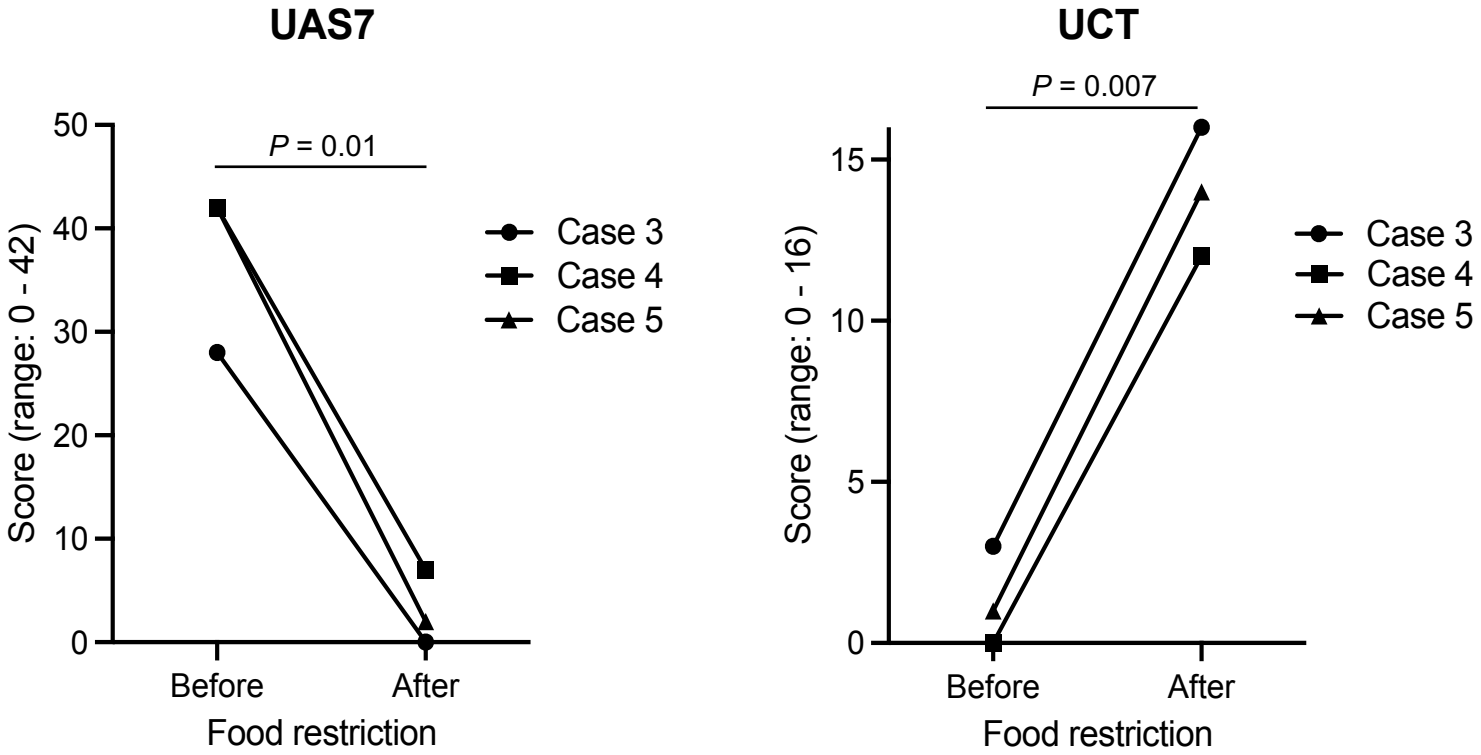
The authors have no conflict of interest to declare.

Authors' contributions

RF, TO and HT designed the study, performed the statistical analysis and interpretation of the results and wrote the manuscript. YA, CS, ST, FYS, HI, MK, ST, AT and MA contributed to data collection. All authors read and approved the final manuscript.

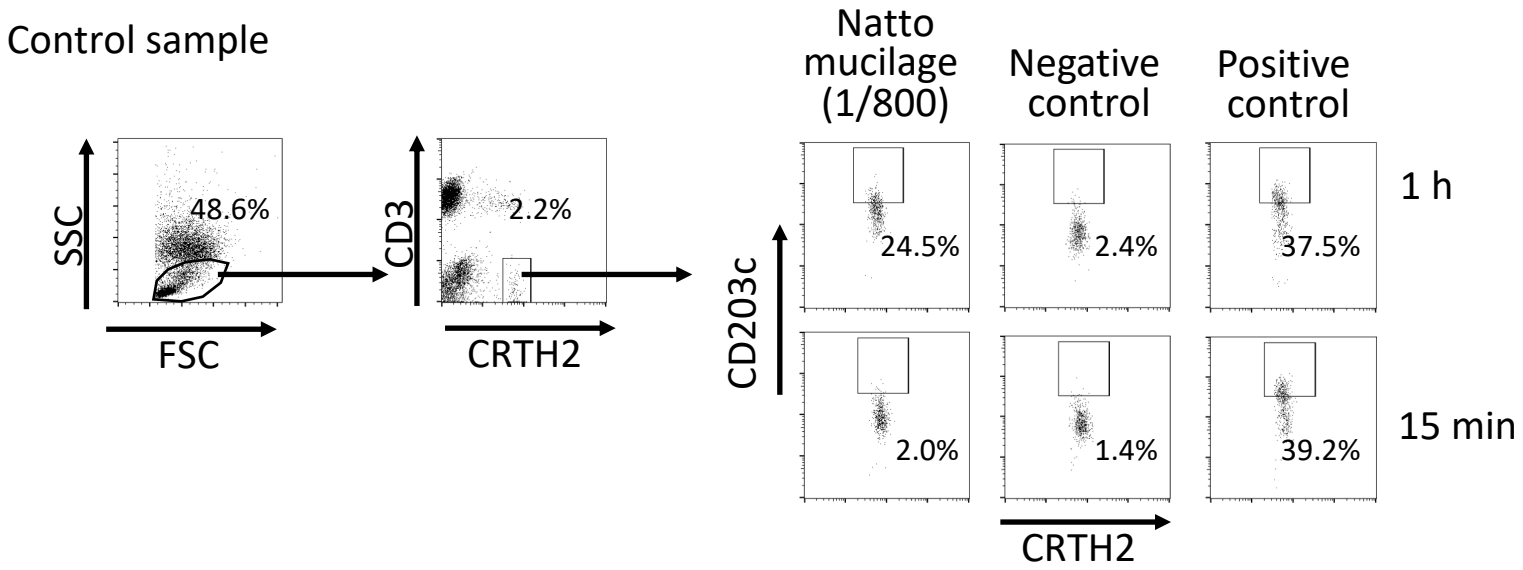
References

1. Parkouda C, Nielsen DS, Azokpota P, Ouoba LI, Amoa-Awua WK, Thorsen L, et al. The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia. *Crit Rev Microbiol* 2009;**35**:139–56.
2. Inomata N, Nomura Y, Ikezawa Z. Involvement of poly (γ-glutamic acid) as an allergen in late-onset anaphylaxis due to fermented soybeans (natto). *J Dermatol* 2012;**39**:409–12.
3. Inomata N, Osuna H, Ikezawa Z. Late-onset anaphylaxis to Bacillus natto-fermented soybeans (natto). *J Allergy Clin Immunol* 2004;**113**:998–1000.
4. Inomata N, Osuna H, Kawano K, Yamaguchi J, Yanagimachi M, Matsukura S, et al. Late-onset anaphylaxis after ingestion of Bacillus Subtilis-fermented soybeans (Natto): clinical review of 7 patients. *Allergol Int* 2007;**56**:257–61.
5. Fukuda R, Ouchi T, Shiiya C, Yasuda-Sekiguchi F, Kouno M, Takahashi S, et al. Urticaria due to natto (fermented soybeans). *Clin Exp Dermatol* 2021;**46**:932–4.
6. Bernstein IL, Li JT, Bernstein DI, Hamilton R, Spector SL, Tan R, et al. Allergy diagnostic testing: an updated practice parameter. *Ann Allergy Asthma Immunol* 2008;**100**:S1–148.
7. Yamakawa K, Inomata N, Fukuro K, Sano S, Nakagawara R, Aihara M. Fermented soybean-induced late-onset anaphylaxis in a 7-year-old junior surfer. *J Dermatol* 2020;**47**:e17–8.
8. Suzuki K, Futamura K, Sato N, Nakamura M, Matsunaga K, Yagami A. Two cases of fermented soybean (natto) allergy diagnosed using the skin prick test and enzyme linked immunosorbent assay for poly-γ-glutamic acid. *J Dermatol* 2020;**47**:429–30.
9. Inomata N, Chin K, Aihara M. Anaphylaxis caused by ingesting jellyfish in a subject with fermented soybean allergy: possibility of epicutaneous sensitization to poly-gamma-glutamic acid by jellyfish stings. *J Dermatol* 2014;**41**:752–3.
10. Shih IL, Van YT. The production of poly-(gamma-glutamic acid) from microorganisms and its various applications. *Bioresour Technol* 2001;**79**:207–25.
11. Chettri R, Bhutia MO, Tamang JP. Poly-γ-Glutamic acid (PGA)-Producing Bacillus species isolated from kinema, Indian fermented soybean food. *Front Microbiol* 2016;**7**:971.
12. Cardona V, Ansotegui IJ, Ebisawa M, El-Gamal Y, Fernandez Rivas M, Fineman S, et al. World allergy organization anaphylaxis guidance 2020. *World Allergy Organ J* 2020;**13**:100472.
13. 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. *Allergy* 2018;**73**:1393–414.
14. Inomata N, Chin K, Nagashima M, Ikezawa Z. Late-onset anaphylaxis due to poly (gamma-glutamic acid) in the soup of commercial cold Chinese noodles in a patient with allergy to fermented soybeans (natto). *Allergol Int* 2011;**60**:393–6.
15. Oda Y, Ikkaku N, Horikawa N, Inomata N. [A case of anaphylaxis due to poly gamma-glutamic acid]. *[Jpn J Clin Dermatol]* 2016;**70**:558–61 (in Japanese).
16. Lund G, Jacobi H, Skov PS, Holm J, Lund K. Effect on cell surface markers following allergen-induced desensitization of human whole-blood basophils. *Int Arch Allergy Immunol* 2010;**153**:323–34.
17. Appel MY, Nachshon L, Elizur A, Levy MB, Katz Y, Goldberg MR. Evaluation of the basophil activation test and skin prick testing for the diagnosis of sesame food allergy. *Clin Exp Allergy* 2018;**48**:1025–34.
18. Koplin JJ, Perrett KP, Sampson HA. Diagnosing peanut allergy with fewer oral food challenges. *J Allergy Clin Immunol Pract* 2019;**7**:375–80.
19. Kumagai T, Yamaguchi N, Hirai H, Kojima S, Kodani Y, Hashiguchi A, et al. Loxoprofen sodium induces the production of complement C5a in human serum. *Int Immunopharmacol* 2016;**33**:55–62.
20. Wensing M, Penninks AH, Hefle SL, Koppelman SJ, Bruijnzeel-Koomen CA, Knulst AC. The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy. *J Allergy Clin Immunol* 2002;**110**:915–20.
21. MacGlashan Jr DW. Basophil activation testing. *J Allergy Clin Immunol* 2013;**132**:777–87.
22. Christensen LH, Holm J, Lund G, Riise E, Lund K. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. *J Allergy Clin Immunol* 2008;**122**:298–304.
23. Cromwick AM, Birrer GA, Gross RA. Effects of pH and aeration on gamma-poly(glutamic acid) formation by Bacillus licheniformis in controlled batch fermentor cultures. *Biotechnol Bioeng* 1996;**50**:222–7.



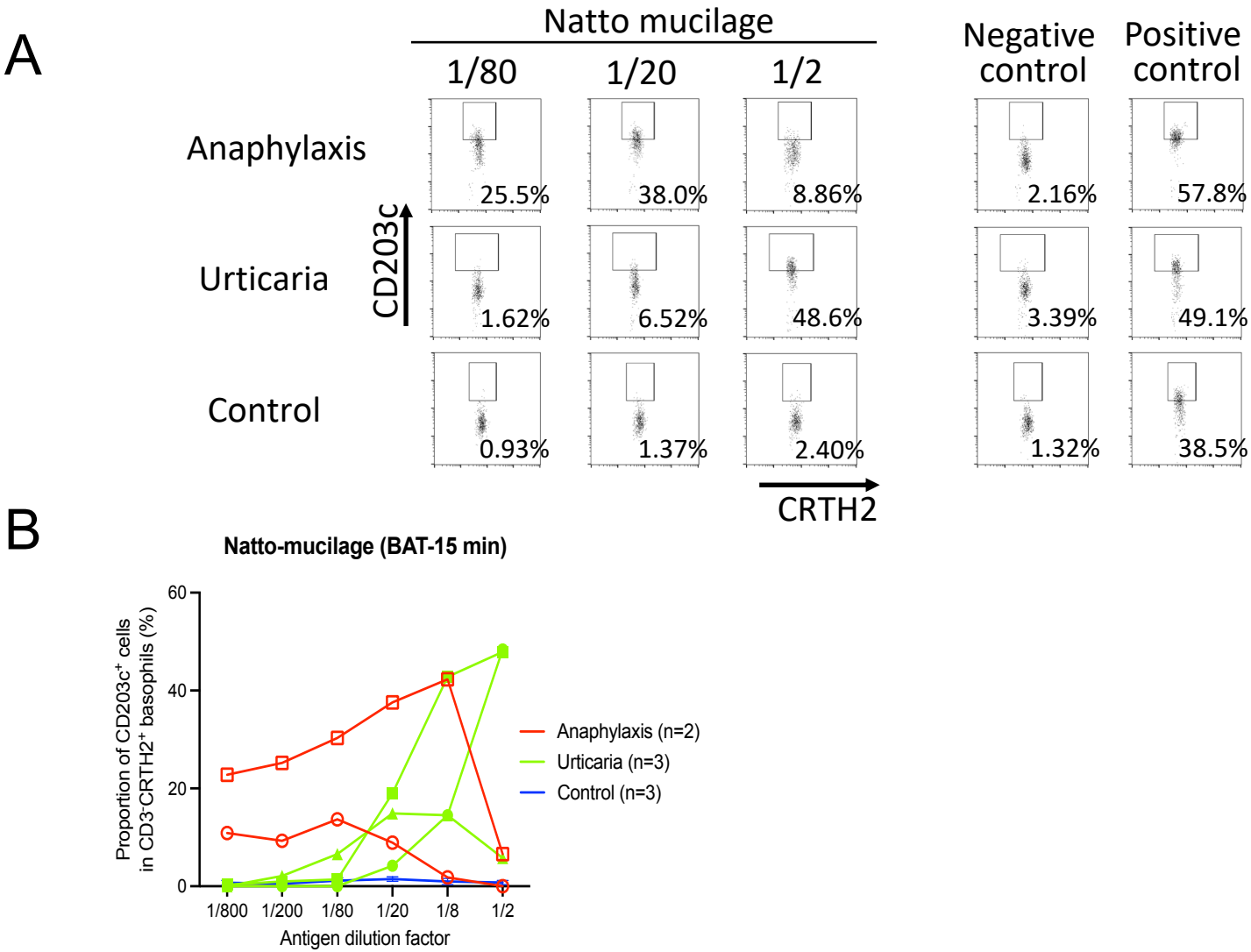
Supplementary Figure. 1. Changes in weekly urticaria activity scores (UAS7) and urticaria control tests (UCTs) before and after restriction of natto and PGA-containing foods.

USA7 and UCT scores were evaluated before and after the restriction of natto and PGA-containing foods in Case 3 (closed circles) ,Case 4 (closed squares) and Case 5 (closed triangles). The range of values in the UAS7 was 0–42 and that of UCT was 16–0.[6] Significant differences between before and after food restriction were evaluated using paired *t* test.



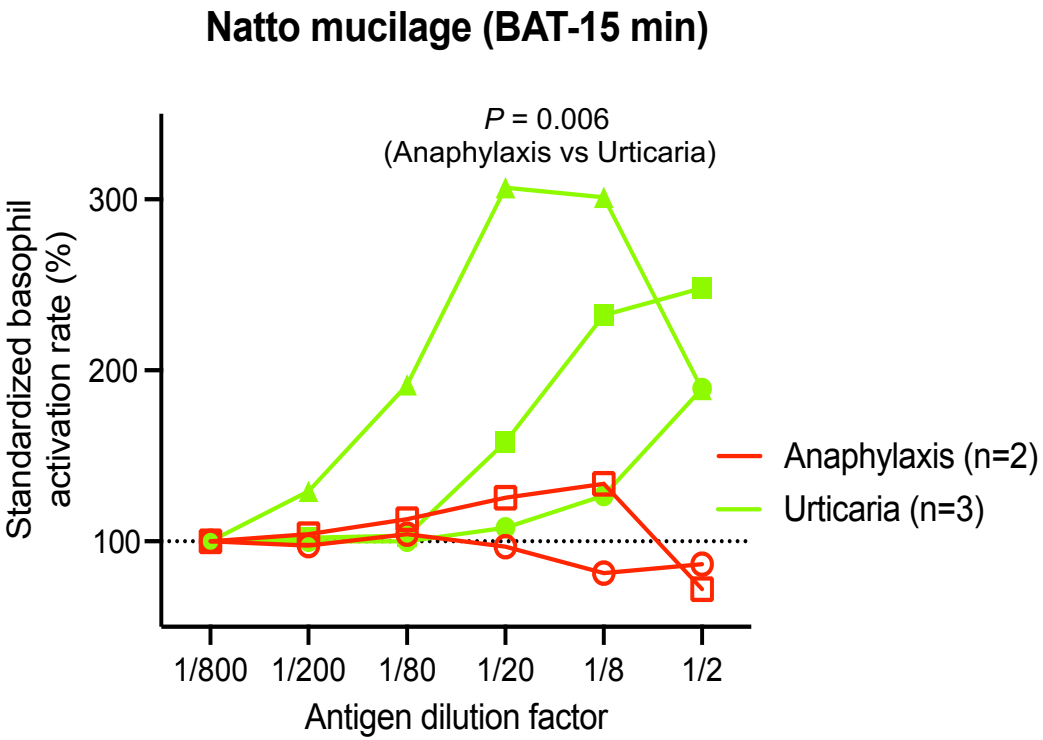
Supplementary Figure. 2. Representative flow cytometry results and gating technique of BAT for 15 min and 1 h incubation time in the control group.

Basophils were defined as CD3-negative and CRTH2-positive population. Proportion of CD203c^{+} cells are measured after gating on CD3^{-}CRTH2^{+} population.



Supplementary Figure. 3. Representative BAT results for natto mucilage in the 15-min condition.

(A) Representative BAT results for natto mucilage. (B) Raw data of BAT for natto mucilage are shown for anaphylaxis cases (red line), urticaria cases (green line), and control patients (blue line). Data from control patients are shown as means ± SEMs.



Supplementary Figure. 4. Results of standardized basophil activation rate for natto mucilage in the BAT under the 15-min condition.

Standardized basophil activation rate for natto mucilage are shown in anaphylaxis cases (red line), urticaria cases (green line). Significant differences between these cases were evaluated using two-way repeated measures ANOVA.