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Letter to Editor

To the Editor

It has been shown that egg-allergic children have higher ovomucoid (OVM)-specific IgD (sIgD) levels compared to atopic controls.¹ Within egg-allergic children, those with higher levels of OVM-sIgD have a decreased risk of anaphylaxis. A study in patients undergoing egg oral immunotherapy (OIT) demonstrated that ovalbumin (OVA)-sIgD levels increase in egg-allergic children desensitized by OIT, but not in children unresponsive to OIT or with sustained unresponsiveness to OVA challenge.² The natural development of tolerance and the acquisition of sustained unresponsiveness due to OIT in egg-allergic children are associated with an increase in OVM-specific IgG4 (sIgG4) levels and a decrease in OVM-specific IgE (sIgE) levels.³ In this cross-sectional study, we elucidated the potential role of IgD in the outgrowing of egg allergy by analyzing egg white (EW)-, OVM-, and OVA-sIgD and sIgG4 levels in sera from 57 egg-allergic children and 23 healthy non-egg allergic children (non-egg allergy: NEA) (Table S1). Of the egg-allergic children, 28 avoided all forms of egg in the diet (complete avoidance of egg: CAE), 18 were able to ingest at least 1/32 cooked whole egg (194mg protein) but not one cooked whole egg (3200 mg protein) (partial avoidance of egg: PAE), and 11 outgrew egg allergy (OGE). The sample size was

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chosen based on similar previous analyses^{1, 4}. The study was approved by The Research Ethics Committee of University of Fukui (#20110052), and written informed consent was obtained from the parent or guardians.

EW- and OVM-sIgE levels measured using ImmunoCAP (Thermo-Fisher Inc., MA) were higher in the CAE group, followed by the PAE, OGE, and NEA groups (Fig 1, Fig S1). The CAE group exhibited lower serum levels of EW- and OVA-sIgD compared to the NEA group and the PAE group, respectively, and had the lowest OVM-sIgD serum levels among all groups, suggesting that OVM-sIgD levels are associated with outgrowing egg allergy. We observed the lowest serum levels of EW-, OVA-, and OVM-sIgG4 in the CAE group, followed by the PAE and OGE groups. The ratio of OVM-sIgD to OVA-sIgD increased as children outgrew egg allergy, while the ratio of OVM-sIgG4 to OVA-sIgG4 did not change (Fig S2). Thus, the production of OVM-sIgD differs from OVM-sIgG4 as children naturally outgrow egg allergy.

High-affinity, but not low-affinity IgE is known to cause anaphylaxis.⁵ High-affinity IgE is derived from memory IgG1⁺ B cells without further affinity maturation, whereas low-affinity IgE is derived from naïve IgM⁺IgD⁺ B cells. Considering class switching pathways and affinity maturation, elevated OVM-sIgD levels might be associated with low-affinity OVM-sIgE levels and reflect the replacement of high-affinity with low-affinity OVM-specific IgE as children outgrow egg allergy, resulting in hypo-responsiveness to OVM.

A recent study found that OVM-sIgE avidity was more effective at differentiating clinically reactive egg-allergic patients from those tolerant of heated egg compared to EW-sIgE.⁴ We found that the ratio of OVM-sIgE to OVM-sIgD or sIgG4 in the CAE group was significantly higher compared to the PAE, OGE, and NEA groups (Fig 2). Receiver operating analysis revealed that the ratio of OVM-sIgE to OVM-sIgD discriminated non-tolerant from partially-tolerant egg-allergic patients with the largest area under the curve (AUC = 0.965) compared with levels of OVM-sIgE or the ratio of OVM-sIgE to OVM-sIgG4. The optimal

cutoff for the ratio of OVM-sIgE to OVM-sIgD had 86.5% sensitivity and 96.4% specificity to identify high-risk subjects (Table S2).

There are several limitations to this study. First, there was a small number of patients. Second, children were only challenged with heated egg and were instructed to avoid egg of any form if they tested positive with less than 1/32 cooked whole egg. Finally, there was a lack of trajectory of sIgD levels during natural tolerance development.

In conclusion, the ratio of OVM-sIgE to OVM-sIgD is a useful marker to identify high-risk egg-allergic patients capable of ingesting a low-dose of cooked whole egg who might be a good candidate for low-dose oral food challenge tests.

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Title:Ovomucoid-specific IgD increases in children who naturally outgrow egg allergy in a cross-sectional study.

Short title:Ovomucoid-specific IgD in egg-allergic children

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Conflict of interest:

The authors declare that they have no conflicts of interest.

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Author contribution:

NI, MY, AK, HM, EN, YH, and KO collected the samples. NI wrote the manuscript and YO organized the study. All authors read and approved the final manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to YO).

Figure legends

Figure 1 Egg allergen-specific IgE, IgD, and IgG4 levels in egg-allergic and non-egg allergic patients (NEA). Ovomucoid (OVM)-specific IgE (sIgE)(A), specific IgD (sIgD)(B), and specific IgG4 (sIgG4)(C), and relationship between ovalbumin (OVA) and OVM-sIgD (D) or sIgG4 (E) in the complete avoidance of egg (CAE), partial avoidance of egg (PAE), outgrown egg allergy (OGE), and NEA groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 2 Relationship between ovomucoid-specific IgD, IgG4, and IgE in egg-allergic and non-egg allergic patients (NEA), and Receiver-operating characteristic analysis predicting tolerance to ingested cooked egg white. The logarithms of the ratio of OVM-sIgE to OVM-sIgD (A), OVM-sIgE to OVM-sIgG4 (B), and OVM-sIgD to OVM-sIgG4 in the complete avoidance of egg (CAE), partial avoidance of egg (PAE), outgrown egg allergy (OGE), and NEA groups. Receiver-operating characteristic curves (C) of OVM-sIgE (long dashed double-dotted line), sIgE/sIgG4 (dotted line), sIgD (broken line), and sIgE/sIgD (bold line). * $p < 0.001$

Figure 2

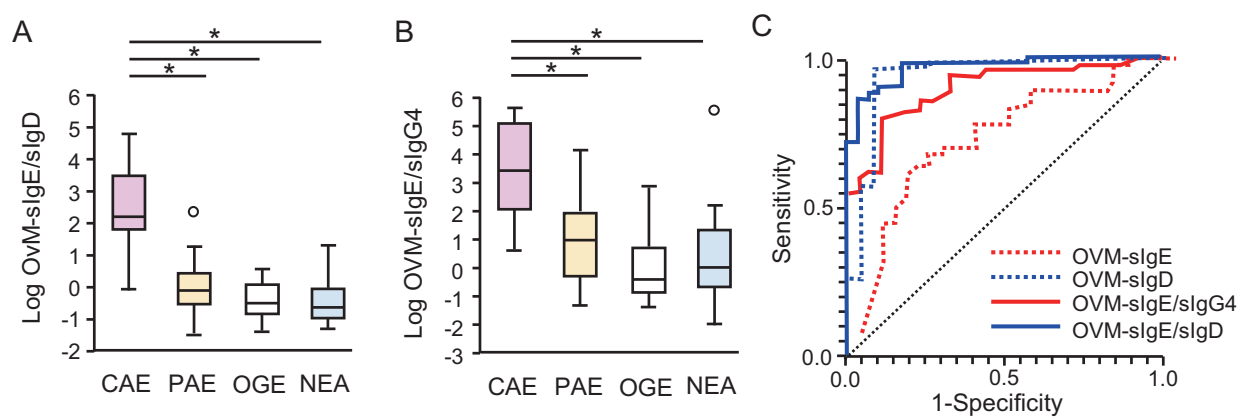


Figure 1

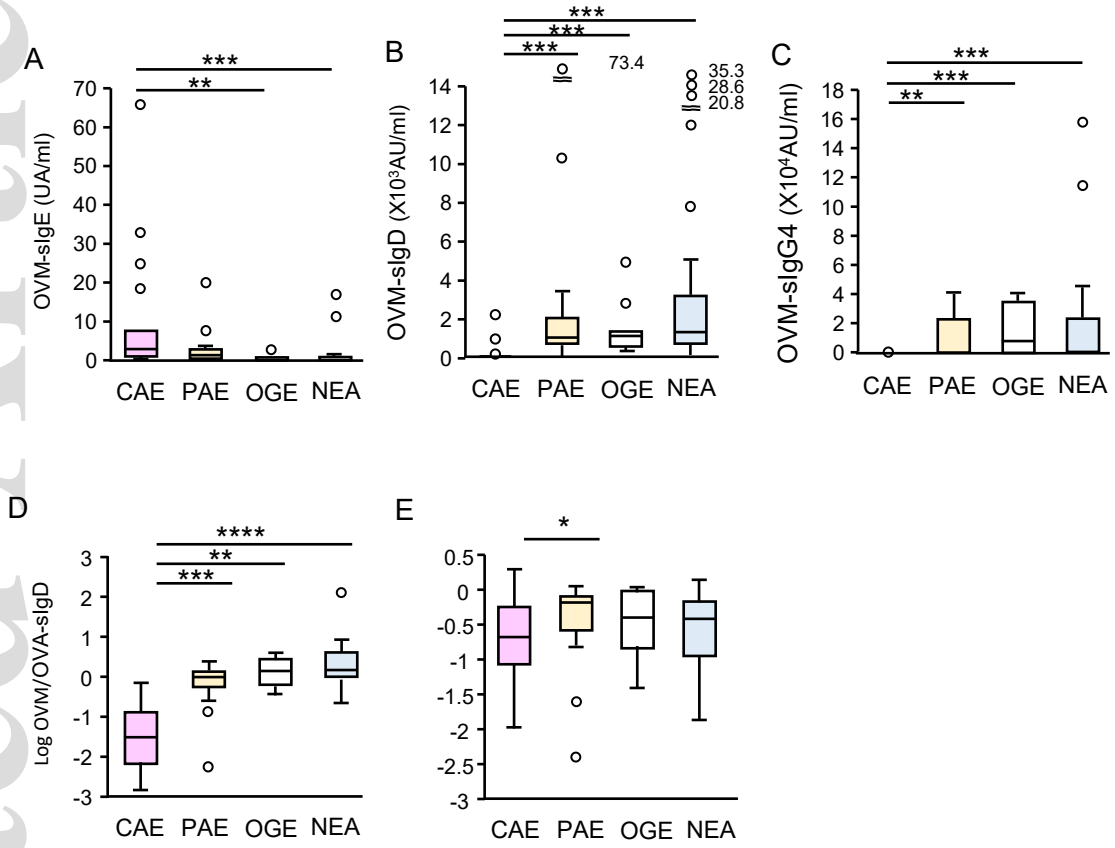


Figure 2

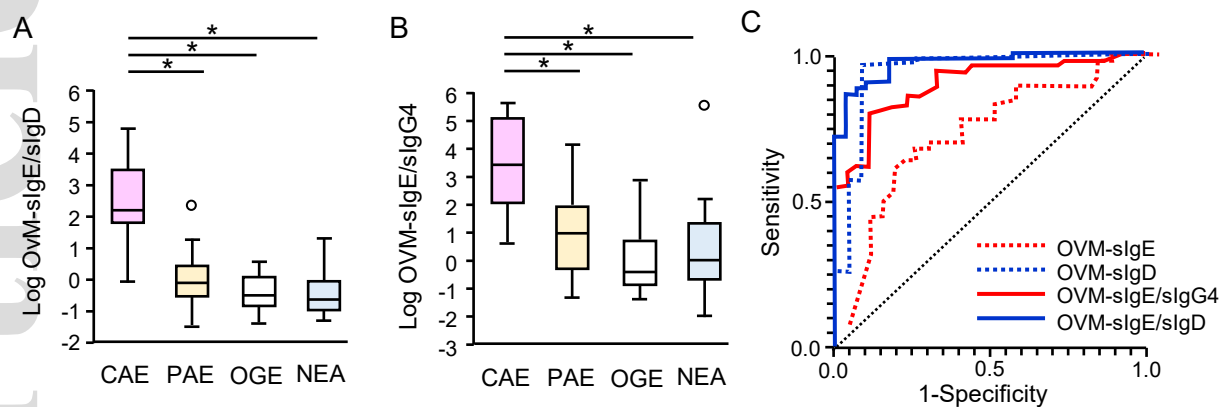


Figure S1

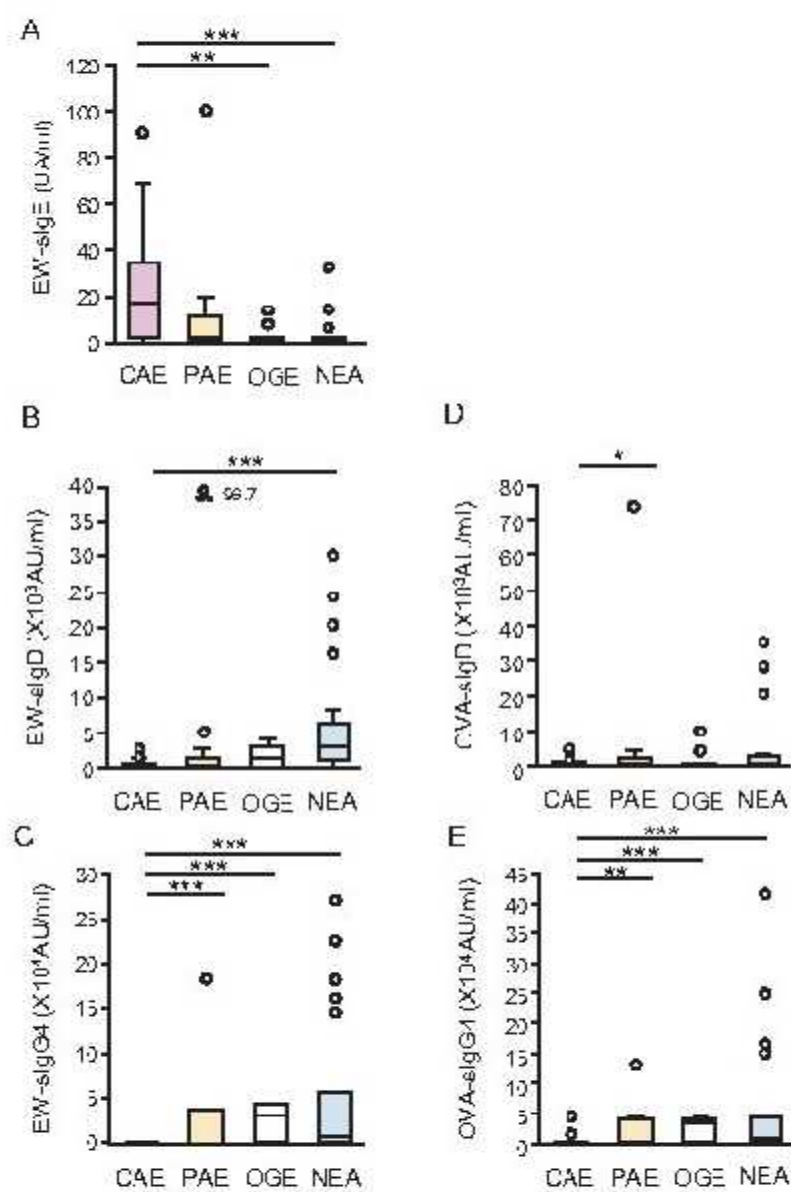


Figure S2

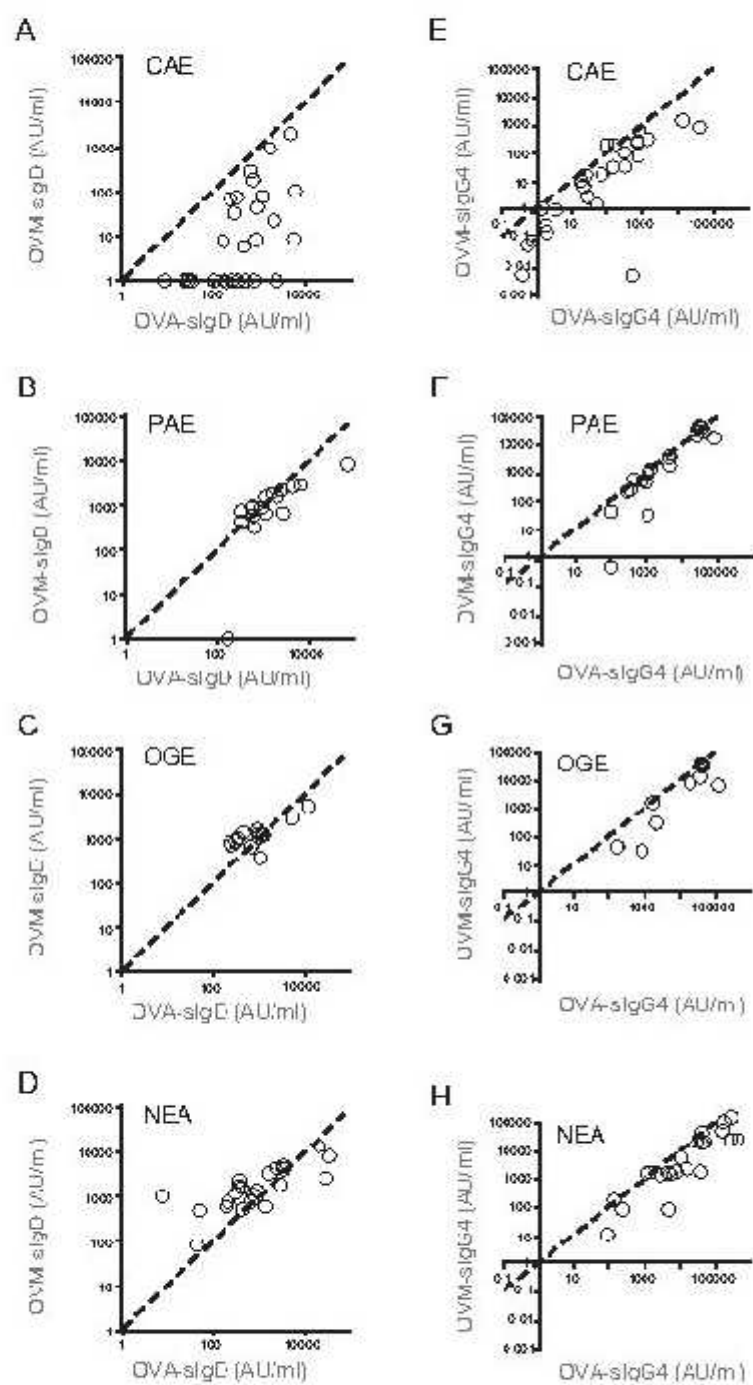


Figure S1

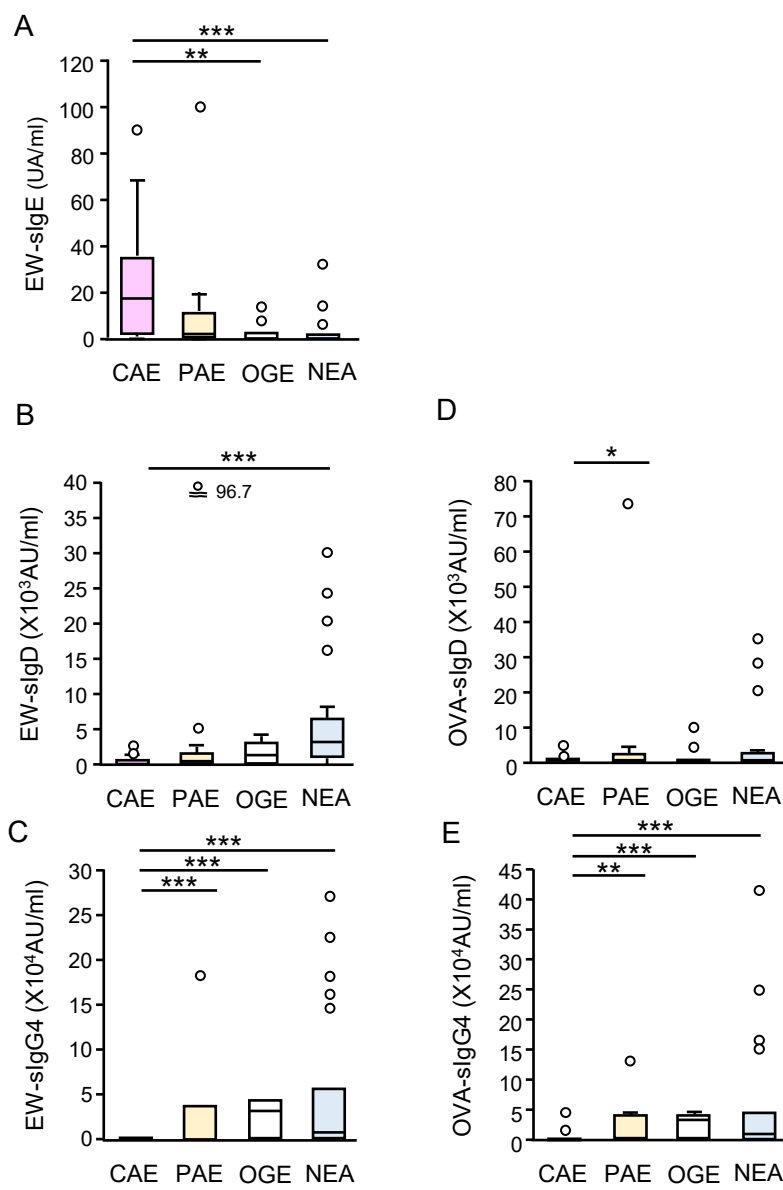
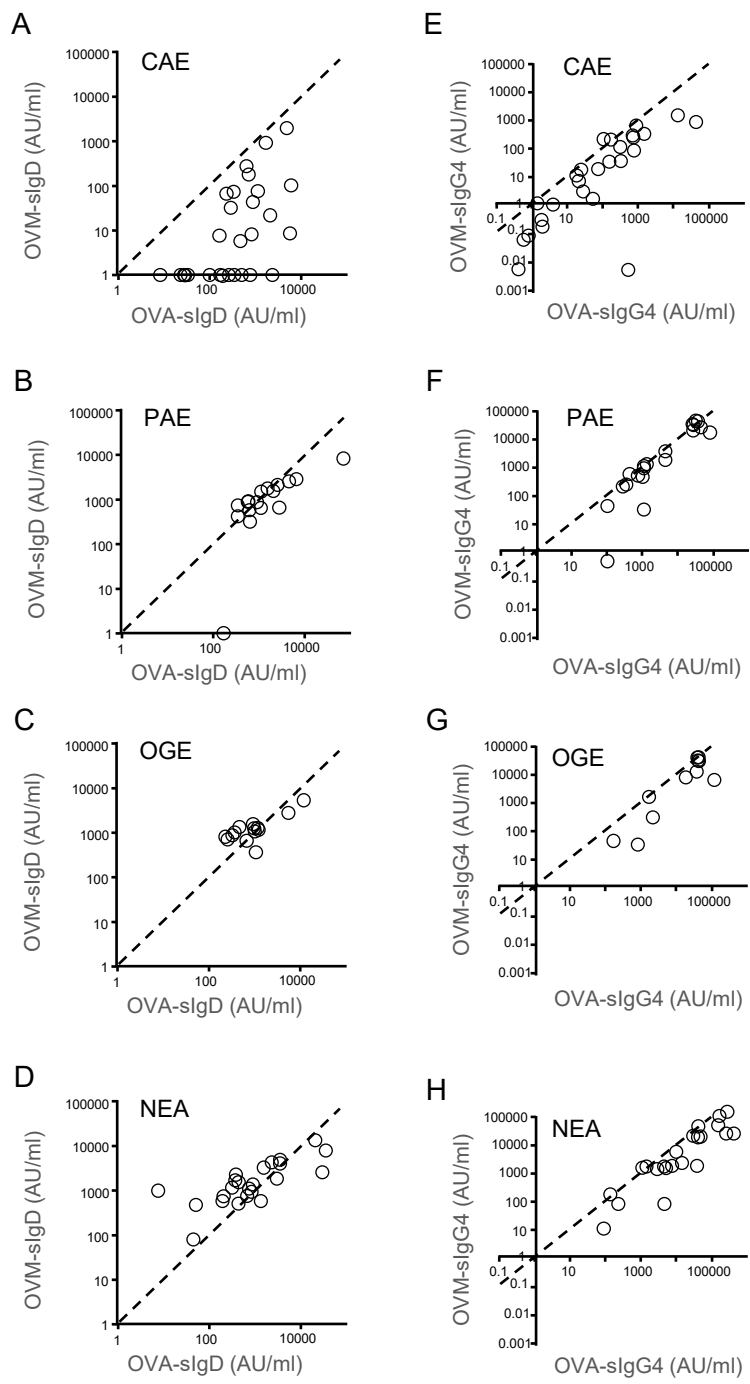


Figure S2



Supporting information

Study participants

Serum samples were collected from 57 egg-allergic and 23 healthy non-egg allergic (NEA) children from September 2019 to August 2020 as a cross-sectional study and stored at -80°C until subsequent analyses. Egg-allergic children with EW-sIgE levels greater than 0.70 kUA/L and apparent allergic reactions after ingestion of egg and egg products were enrolled in this study. The tolerated egg dose for each patient was determined by oral food challenge and/or an episode of accidental ingestion of cooked EW within one month of blood sampling. Egg-allergic patients were classified into three groups: 28 patients avoided all forms of egg in the diet (complete avoidance of egg: CAE), 18 patients were able to ingest at least 1/32 cooked whole egg (low dose; 194 mg protein) but not one cooked whole egg (full dose; 3200 mg protein) (partial avoidance of egg: PAE), and eleven patients outgrew egg allergy (OGE). Patients in the PAE group were allowed to eat baked egg products containing the same egg protein dose as tolerated cooked egg. Healthy children who had no episode of allergic reactions after ingestion of egg and egg products were enrolled in the NEA group.

Yanagida et al. reported that low-dose OIT with 1/32 of a cooked whole egg induced sustained unresponsiveness to 1/32 and 1/2 of a cooked whole egg, with no severe symptoms, demonstrating

that low-dose oral food challenge and oral immunotherapy could accelerate the tolerance to causative foods^{1,2}. Based on their report, Japanese food allergy guidelines recommend an oral food challenge test using a low cumulative dose, corresponding to 1/32 of a cooked whole egg in high-risk cases where a small dose may induce symptoms³. Therefore, we used 1/32 of a cooked whole egg as a cutoff volume to discriminate CAE and PAE groups.

Measurement of allergen-specific Ig levels

Serum levels of EW-, OVM-, and OVA-sIgD and sIgG4 were determined by ELISA. In brief, microtiter plates (Nalgen Nunc International Co. Tokyo, Japan) were coated with 100 μ L/well of EW, OVM, or OVA (100 μ g /mL) overnight at 4°C and then were blocked by incubation with ELISA blocking reagent (Roche Diagnostics Co., Tokyo, Japan) for 3 hours at room temperature. After washing four times with PBS/0.05% Tween 20, the plates were incubated with 100 μ L/well of diluted serum samples overnight at 4°C. After washing four times with PBS/0.05% Tween 20, plates were then incubated with 100 μ L/well of biotin-conjugated rat anti-human IgD or IgG4 antibody (Southern Biotech, AL) at 2 μ g/mL in PBS/0.05% Tween 20, followed by washing four times with PBS/0.05% Tween 20 and 2-hours incubation with 100 μ L/well of horseradish peroxidase-conjugated streptavidin (X4000 in PBS/0.05% Tween 20, Thermo-Fisher Inc.). The plates were developed with the TMB microwell peroxidase substrate system (Kirkegaard and

Perry Laboratories Inc., Guildford, UK). The antigen-specific antibody titers in the serum were expressed as arbitrary units determined using the standard pooled serum, which contained high titers of antigen-specific antibody.

Statistical analysis

Statistical analysis was performed using GraphPad Prism ver 8.4.1(GraphPad Software, San Diego, CA). Kruskal-Wallis test and Dunn's multiple comparison test were used to compare the antigen-specific Ig levels of each group. The correlation between antigen-sIgE, sIgD, and sIgG4 levels was analyzed by Spearman's rank correlation test. A p-value < 0.05 was considered statistically significant.

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58

59 **Table S1. Subject demographics**

	CAE	PAE	OGE	NEA	<i>p</i> -value
Number	28	18	11	23	
Male (%)	24 (85.7%)	9 (50%)	7 (63.6%)	10 (43.5%)	0.02
Age (Years)	1 (1-2.25)	3 (2-4.5)	2 (1.5-3.5)	3 (1-4)	n.s.
Onset age of egg allergy (Years)	0.75 (0.5-0.9)	0.83 (0.67-0.98)	0.67 (0.50-0.71)		n.s.
AD (%)	18 (64.3%)	12 (66.7%)	7 (63.6%)	11 (47.8%)	n.s.
Eczema (%)	11 (39.3%)	9 (50.0%)	6 (54.5%)	10 (43.5%)	n.s.
Family AD (%)	17 (60.7%)	12 (66.7%)	4 (36.4%)	7 (30.4%)	0.0127
WBC (/μL)	11,200 (8,300-14,800)	7,350 (6,300-8,925)	8,500 (7,100-9,800)	8,600 (7,500-11,300)	n.s.
Eo (%)	3 (2-4.2)	3 (1.9-4.3)	2 (1.3-4)	2 (1-3)	n.s.
Plt (X10 ⁴ /μL)	34.7 (30.6-43.4)	29.2 (27.0-35.4)	35.4 (29.1-38.1)	33.7 (26.7-35.7)	n.s.
Total IgE (IU/mL)	158 (47.5-522.5)	140.5 (50.8-452.3)	60.5 (48-440)	22.3 (8.7-110.7)	n.s.
EW-sIgE (UA/mL)	18.6 (2.9-35.5)	2.0 (1.1-6.2)	1.48 (0.98-2.59)	0.9 (0.3-1.4)	<0.0001
OVM-sIgE (UA/mL)	3.30 (1.2-10.3)	1.48 (0.5-2.1)	0.48 (0.3-1.1)	0.36 (0.26-1.11)	<0.0001

60 Data are shown as median (range). AD, Allergic diseases; WBC, White blood cells; Eo, Eosinophils,
61 Plt, Platelet; sIgE, antigen-specific immunoglobulin E; EW, egg-white; OVM, ovomucoid; CAE,
62 complete avoidance of egg; PAE, partial avoidance of egg; OGE, outgrown egg allergy; NEA, non-
63 egg allergy; n.s., not significant

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Table S2 Optimal cutoffs to classify subjects reacting to a low dose of heated egg.

Parameters	AUC (95% CI)	Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
EW-sIgE	0.822 (0.732-0.911)	1.565	55.8	96.4	96.7	54.0
OVM-sIgE	0.774 (0.667-0.881)	0.650	57.7	85.7	88.2	52.2
OVM-sIgD	0.941 (0.876-1.000)	0.317	96.2	13.5	96.2	92.9
OVM-sIgG4	0.864 (0.787-0.941)	0.094	69.2	96.4	97.3	62.8
OVM-sIgE/sIgD	0.965 (0.930-1.000)	0.468	86.5	96.4	97.8	79.4
OVM-sIgE/sIgG4	0.899 (0.831-0.968)	1.848	78.8	89.3	93.2	69.4

Optimal cutoffs were determined based on the Youden index, which is the distance between the point of inflection of the receiver operator characteristic curve and the reference line. Sensitivity, specificity, PPV, and NPV with 95% CIs are indicated for each cutoff.

NPV, Negative predictive value; PPV, positive predictive value.

Figure legends

Figure S1 Egg allergen-specific IgE, IgD, and IgG4 levels in egg-allergic and non-egg allergic patients (NEA). Egg white (EW)- (A, B, and C), and ovalbumin (OVA)- (D and E) specific IgE (sIgE)(A), specific IgD (sIgD)(B and D), and specific IgG4 (sIgG4)(C and E) in the complete avoidance of egg (CAE), partial avoidance of egg (PAE), outgrown egg allergy (OGE), and NEA groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure S2 Relationship between ovalbumin (OVA)- and ovomucoid (OVM)-specific IgD or IgG4 in egg-allergic and non-egg allergic patients (NEA). Relationship between OVA- and OVM-sIgD (A, B, C, and D) or sIgG4 (E, F, G, and H) in the complete avoidance of egg (CAE) (A and E), partial avoidance of egg (PAE) (B and F), outgrown egg allergy (OGE) (C and G), and NEA (D and H) groups. * $p < 0.05$, ** $p < 0.001$