Published in final edited form as:

J Allergy Clin Immunol Pract. 2018 ; 6(3): 1070–1072.e4. doi:10.1016/j.jaip.2017.10.037.

Underestimation of Specific IgE Measurements Using Extract-Based Assays on Undiluted Sera Revealed through Dilution

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To the Editor

The measurement of specific IgE (sIgE) to foods is an established clinical tool for the diagnosis and management of allergic disease.¹ Normally, sIgE is measured using undiluted serum, and assays are only performed at a dilution if the value is ≥ 90 international units (IU)/mL. Thus, when serum gives sIgE values of ≤ 50 IU/mL, the possibility that the value is a significant underestimate is rarely considered. The underestimation of results using undiluted serum is a well-known limitation of immunoprecipitation, immunodiffusion, and nephelometry, where it is referred to as a “prozone” or “hook” effect.² However, there is very little awareness of this phenomenon in the measurement of sIgE.

We sought to investigate this phenomenon using sera collected from patients with the α -gal syndrome (AS) (n=3), which involves delayed reactions to mammalian products (red meat or dairy) related to sIgE to galactose- α -1,3-galactose (α -gal).^{3,4} We addressed additional allergens using sera from subjects with eosinophilic esophagitis (EoE) (n=17), peanut allergy (PA) (n=4) or oral allergy syndrome (OAS) (n=5). Serum was collected from patients at The University of Virginia (Charlottesville, VA) or The Ohio State University/Nationwide Children's Hospital (Columbus, Ohio) clinics. We measured sIgE to beef, milk, mite (*Dermatophagoides pteronyssinus*), cat dander, cow's milk, and peanut extracts by ImmunoCAP in progressively diluted sera. The results were expressed in fluorescent response units (FRU) or IU/mL (positive ≥ 0.35 IU/mL). In addition sIgE was measured to purified allergens by ImmunoCAP (α -gal, Bos d 4, Bos d 5, Bos d 6, Bos d 8, Ara h 1, Ara h 2, and/or Ara h 8) or Immuno Solid-Phase Allergen Chip (ISAC) (Der p 1, Der p 2, Der p

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10, Fel d 1, and/or Fel d 4) to determine the patients' dominant sensitization(s). Methods for sIgE to α -gal, ISAC, and IgG₄ assays are detailed in the Supplementary Materials. In brief, the α -gal sIgE assay uses 20 μ g cetuximab coupled to the solid phase.

The result in Fig 1A demonstrates that there is a substantial dilution effect when assaying sIgE to beef and milk extract in sera from a subject with AS. Of note, α -gal constitutes a minor fraction of the antigens in beef and milk. To further examine this phenomena we performed dilution studies using ImmunoCAP for beef extract or α -gal on sera from three AS patients who had highly-positive sIgE to α -gal (>100 IU/mL). These values for sIgE to α -gal allowed us to dilute the sera past 1:1000 before sIgE levels were undetectable. We compared curves for sIgE to beef extract and α -gal to a control curve, which revealed dramatic differences [Fig 1B and 1C]. Focusing on sIgE to beef extract in AS #1, the FRU in the undiluted serum were approximately the same as the value for the 1:4 dilution, which illustrated a "hook" effect [Fig 1C].

In another AS patient who was highly-positive for sIgE to α -gal (>100 IU/mL), we recreated the situation of insufficient antigen using progressively diluted serum and low cetuximab (800 ng) on the solid-phase [see supplement Fig s1]. The dilution curve deviated from the control curve and that of the standard cetuximab immunoCAP, indicating that the amount of antigen on the solid-phase was insufficient for accurate measurement of sIgE to α -gal. We also applied decreasing quantities of cetuximab to the solid-phase and measured sIgE to α -gal using undiluted serum from an AS patient [Fig s2] and the resultant curve showed a dose-response relationship between the mass of cetuximab on the solid-phase and the assay readout. Thus, manipulating the amount of allergen on the solid-phase, such that there is an imbalance in antibody and allergen concentrations, can markedly change the measured sIgE values.

Next, we performed dilution studies using sIgE assays for mite (n=6), cat dander (n=4), and cow's milk (n=11) extracts in selected IgE-positive sera from 17 EoE patients and also for peanut extract in the sera from the PA and OAS patients. Mite, cat dander, cow's milk, and peanut extracts consist of many different allergens, which are present at unequal amounts on the solid-phase [Table E1]. In cases where Der p 2, Fel d 1, Bos d 8, or Ara h 2 were the primary allergen targets, dilution had little effect on the results; each of these allergens represent a significant or large proportion of the respective extracts [Fig 1D]. In contrast, there was a pronounced dilution effect in many of the sera from subjects dominantly sensitized to Bos d 4 and Ara h 8, which are qualitatively a minor fraction of the relevant extract [Fig 1D and Table E1]. We then determined the percentage difference in sIgE values in the undiluted and 1:8 (calculated) samples from each assay [Table I]. There was a strong negative correlation ($r_s=-0.79$ [95% confidence interval -0.90 to -0.61]; $P<0.001$) between the representation of the dominant allergen for each patient and the percentage difference between the undiluted and 1:8 measurements [Fig 1E and Table E2].

There are two major effects related to the use of undiluted serum in sIgE assays that can cause false negatives or that can underestimate measurements. Both effects essentially occur because of inadequate antigen on the solid-phase. The first effect can occur when using sIgE microarrays (<1 ng of allergen) to measure sera that contains high levels of competing IgG,

such as in patients on allergen immunotherapy or in those with EoE.⁵⁻⁸ The second effect occurs because the relevant protein or oligosaccharide allergen represents only a minor fraction of the total molecules present in the extract. Of note the two effects are not mutually exclusive. EoE is distinct from the other allergic diseases studied here because it is associated with high serum food-specific IgG₄.⁶ However, IgG or IgG₄ blockade is unlikely to be a complete explanation for the dilution effect because there was no correlation between IgG₄:IgE ratios and the dilution effect in EoE sera [Table E3]. Additionally, the dilution curves for sIgE to α -gal in the AS patients behaved properly despite elevation of serum α -gal-specific IgG₁ and IgG₂.⁹

Taken together the results reported here imply that using undiluted serum for sIgE assays that employ heterogeneous antigen may significantly underestimate results if the relevant allergen has minor representation in the extract applied to the solid-phase. Two approaches can be considered to address this possibility, which might be particularly important when there is high clinical suspicion for allergy but an unremarkable IgE result from an extract assay. Component tests, if available, are one option, but simply doing a dilution may be equally helpful to identify the presence of a minor allergen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health [R01-AI-20565].

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Clinical Implications

In clinical practice and most research studies, allergen-specific IgE assays are performed on undiluted sera. However, if the relevant allergen is a minor proportion of the extract on the solid-phase, these assays may seriously underestimate the concentration of specific IgE.

Figure 1.

[A] Sera from a subject positive for AS was assayed in duplicate in dilution series using ImmunoCAP for cetuximab, beef extract and milk extract. The demonstrated response represents the value calculated as corrected for the relevant dilution. Dilution studies using sera from three different AS patients using [B] cetuximab and [C] beef extract and reported as FRU. [D] Representative dilution series using sera from subjects sensitized to milk, peanut, cat or dust mite. [E] Spearman rank correlation between estimated representation of dominant allergen in extract and the percentage difference between sIgE values measured in undiluted sera and those calculated from the 1:8 dilution. Results are from sera from EoE (n=17), PA (n=4), and OAS (n=5) patients with sIgE to mite, cat dander, cow's milk, and peanut extracts, and are grouped by dominant sensitization to Der p 1, Der p 2, Fel d 1, Bos d 4, Bos d 5, Bos d 6, Bos d 8, Ara h 1, Ara h 2 or Ara h 8.

Table 1

Percentage difference in sIgE values between the undiluted and 1:8 diluted sera

Patient	Allergen Extract	sIgE in Undiluted Sample (IU/mL)	sIgE in 1:8 Sample, calculated (IU/mL)	Percentage Difference	Dominant Allergen
Results where the dominant allergen represents a significant proportion of the extract					
EoE #1	Mite	3.58	4.24	+18%	Der p 1
EoE #2	Mite	6.43	7.20	+12%	Der p 2
EoE #3	Mite	10.9	10.9	0%	Der p 2
EoE #4	Mite	14.8	15.0	+1%	Der p 2
	Cat Dander	5.47	6.24	+14%	Fel d 1
EoE #5	Mite	16.2	16.2	0%	Der p 2
	Mite	28.0	31.5	+13%	Der p 2
EoE #6	Cat Dander	3.52	3.84	+9%	Fel d 1
	Cow's Milk	7.29	7.52	+3%	Bos d 8
EoE #7	Cat Dander	8.17	8.72	+7%	Fel d 1
EoE #8	Cat Dander	12.8	13.0	+2%	Fel d 1
EoE#12	Cow's Milk	12.3	13.2	+7%	Bos d 5
EoE#13	Cow's Milk	13.0	15.8	+22%	Bos d 5
EoE#16	Cow's Milk	28.8	33.2	+15%	Bos d 8
PA #1	Peanut	28.9	32.2	+11%	Ara h 2
PA #2	Peanut	42.0	44.1	+5%	Ara h 2
PA #3	Peanut	49.5	52.4	+6%	Ara h 2
PA #4	Peanut	98.0	97.2	-1%	Ara h 2
Results where the dominant allergen represents a minor proportion of the extract					
AS #1	Beef	30.1	73.2	+143%	α-gal
AS #2	Beef	10.7	55.0	+414%	α-gal
AS #3	Beef	39.5	285	+622%	α-gal
EoE #5	Cow's Milk	9.82	14.4	+47%	Bos d 6
EoE #9	Cow's Milk	2.33	8.96	+285%	Bos d 6
EoE#10	Cow's Milk	6.12	37.7	+516%	Bos d 6
EoE#11	Cow's Milk	6.81	27.6	+296%	Bos d 4

Patient	Allergen Extract	sigE in Undiluted Sample (IU/mL)	sigE in 1:8 Sample, calculated (IU/mL)	Percentage Difference	Dominant Allergen
EoE#14	Cow's Milk	13.8	25.8	+87%	Bos d 4
EoE#15	Cow's Milk	15.8	25.8	+209%	Bos d 6
EoE#17	Cow's Milk	35.2	45.7	+30%	Bos d 4
OAS #1	Peanut	4.19	8.64	+106%	Ara h 8
OAS #2	Peanut	4.23	7.20	+70%	Ara h 8
OAS #3	Peanut	8.65	12.0	+39%	Ara h 8
OAS #4	Peanut	8.97	15.4	+72%	Ara h 8
OAS #5	Peanut	17.0	38.3	+125%	Ara h 8