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Clinical Communications

Underestimation of specific IgE measurements using extract-based assays on undiluted sera revealed through dilution

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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.

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 greater than or equal to 90 international units (IU)/mL. Thus, when serum gives sIgE values of less than or equal to 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 ($n = 4$), or oral allergy syndrome ($n = 5$). Serum was collected from patients at the University of Virginia (Charlottesville, Va) or 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 to purified allergens was measured 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 (Der p 1, Der p 2, Der p 10, Fel d 1, and/or Fel d 4) to determine the patients' dominant sensitization(s). Methods for sIgE to α -gal, Immuno Solid-Phase Allergen Chip, and IgG₄ assays are detailed in this article's Online Repository at www.jaci-inpractice.org. In brief, the α -gal sIgE assay uses 20 μ g cetuximab coupled to the solid phase.

The result in Figure 1, A, 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 3 patients with AS 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 (Figure 1, B and C). 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 (Figure 1, C).

In another patient with AS 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 Figure E1 in this article's Online Repository at www.jaci-inpractice.org). 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 a patient with AS (see Figure E2 in this article's Online Repository at www.jaci-inpractice.org) 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 patients with EoE and also for peanut extract in the sera from patients with peanut allergy and oral allergy syndrome. Mite, cat dander, cow's milk, and peanut extracts consist of many different allergens, which are present in unequal amounts on the solid phase (see Table E1 in this article's Online Repository at www.jaci-inpractice.org). In cases in which 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 represents a significant or large proportion of the respective extracts (Figure 1, D). 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 quantitatively a minor fraction of the relevant extract (Figure 1, D; 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% CI, -0.90 to -0.61 ; $P < .001$) between the representation of the dominant allergen for each patient and the percentage difference between the undiluted and 1:8 measurements (Figure 1, E; see Table E2 in this article's Online Repository at www.jaci-inpractice.org).

There are 2 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

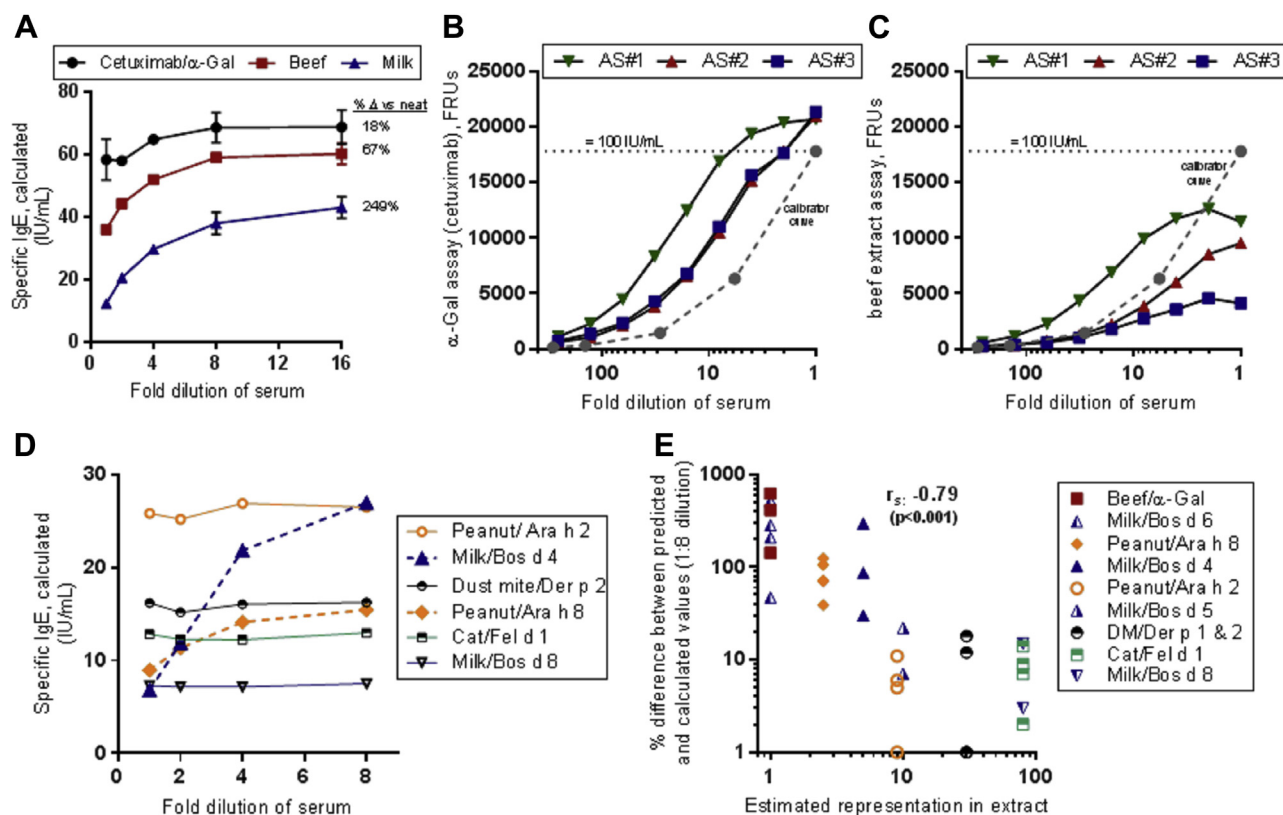


FIGURE 1. A, Sera from a subject positive for AS was assayed in duplicate in dilution series using ImmunoCAP for cetuximab, beef extract, and milk extract. The demonstrated response represents the value calculated as corrected for the relevant dilution. Dilution studies using sera from 3 different patients with AS using (B) cetuximab and (C) beef extract and reported as FRU. D, Representative dilution series using sera from subjects sensitized to milk, peanut, cat dander, 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 patients with EoE ($n = 17$), PA ($n = 4$), and OAS ($n = 5$) 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. OAS, Oral allergy syndrome; PA, peanut allergy.

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 2 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 (see Table E3 in this article's Online Repository at www.jaci-inpractice.org). In addition, the dilution curves for sIgE to α-gal in the patients with AS 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 use 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.

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TABLE I. 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
EoE #6	Mite	28.0	31.5	+13%	Der p 2
	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
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

OAS, Oral allergy syndrome; PA, peanut allergy.

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METHODS

Assays for sIgE to α -gal were performed by coupling biotinylated cetuximab to a streptavidin solid phase.^{E1} Sera from patients with EoE who were positive for sIgE to *Dermatophagoides pteronyssinus* and cat dander extract were assayed for sIgE to 112 molecular allergens by Immuno Solid-Phase Allergen Chip (ISAC) to determine the dominant sensitization. The

results for ISAC were expressed in ISAC standard units. Sera from patients with EoE who were positive for sIgE to cow's milk extract were additionally assayed for specific IgG₄ (sIgG₄) to cow's milk proteins (Bos d 4, Bos d 5, Bos d 6, or Bos d 8) by ImmunoCAP. The results for sIgG₄ were expressed in $\mu\text{g/mL}$ (positive sIgG₄ $\geq 0.07 \mu\text{g/mL}$).

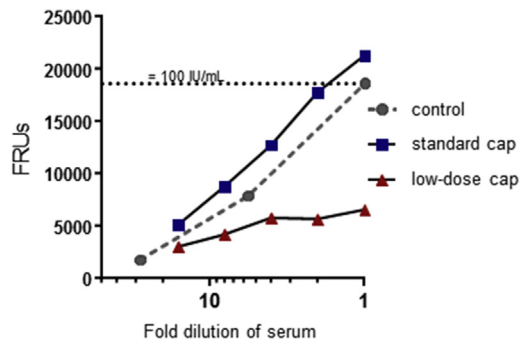


FIGURE E1. Dilution series conducted using sera from a subject with AS using standard cetuximab ImmunoCAP (20 μ g added to solid phase) versus low-dose cetuximab ImmunoCAP (0.8 μ g added to solid phase).

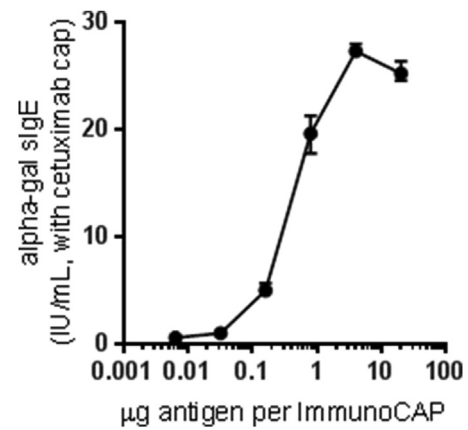


FIGURE E2. Dose response using ImmunoCAPs with varying concentrations of cetuximab on the solid phase. The experiment was done in triplicate with undiluted serum from a subject with AS.

TABLE E1. Analysis of sIgE responses to peanut, *Dermatophagoides pteronyssinus* (mite), cat dander, and cow's milk extracts in undiluted sera from patients with EoE (n = 17), PA (n = 4), and OAS (n = 5)

Patient	<i>D pteronyssinus</i> (mite)			Cat dander		Cow's milk				Peanut		
	Der p 1 (ISU)	Der p 2 (ISU)	Der p 10 (ISU)	Fel d 1 (ISU)	Fel d 4 (ISU)	Bos d 4 (IU/mL)	Bos d 5 (IU/mL)	Bos d 6 (IU/mL)	Bos d 8 (IU/mL)	Ara h 1 (IU/mL)	Ara h 2 (IU/mL)	Ara h 8 (IU/mL)
EoE #1	7.93	1.62	0.00	—	—	—	—	—	—	—	—	—
EoE #2	2.55	2.76	0.00	—	—	—	—	—	—	—	—	—
EoE #3	2.04	5.55	0.00	—	—	—	—	—	—	—	—	—
EoE #4	0.00	10.1	0.00	17.6	0.00	—	—	—	—	—	—	—
EoE #5	12.0	25.8	0.96	—	—	<0.35	<0.35	13.1	1.69	—	—	—
EoE #6	7.79	24.4	0.00	6.34	0.00	1.80	1.02	<0.35	6.82	—	—	—
EoE #7	—	—	—	17.4	0.00	—	—	—	—	—	—	—
EoE #8	—	—	—	16.4	0.00	—	—	—	—	—	—	—
EoE #9	—	—	—	—	—	<0.35	0.57	1.75	0.54	—	—	—
EoE #10	—	—	—	—	—	3.73	3.13	5.07	1.51	—	—	—
EoE #11	—	—	—	—	—	9.05	3.14	1.97	0.77	—	—	—
EoE #12	—	—	—	—	—	1.98	6.80	0.44	6.34	—	—	—
EoE #13	—	—	—	—	—	1.92	15.2	1.22	3.62	—	—	—
EoE #14	—	—	—	—	—	14.2	2.31	<0.35	2.82	—	—	—
EoE #15	—	—	—	—	—	3.02	7.03	42.0	4.51	—	—	—
EoE #16	—	—	—	—	—	0.53	2.63	15.1	24.2	—	—	—
EoE #17	—	—	—	—	—	30.5	4.86	3.13	24.0	—	—	—
PA #1	—	—	—	—	—	—	—	—	—	0.48	28.6	<0.35
PA #2	—	—	—	—	—	—	—	—	—	24.4	40.2	1.00
PA #3	—	—	—	—	—	—	—	—	—	23.8	39.4	<0.35
PA #4	—	—	—	—	—	—	—	—	—	15.6	48.2	<0.35
OAS #1	—	—	—	—	—	—	—	—	—	<0.35	1.28	32.7
OAS #2	—	—	—	—	—	—	—	—	—	<0.35	<0.35	15.3
OAS #3	—	—	—	—	—	—	—	—	—	<0.35	<0.35	12.1
OAS #4	—	—	—	—	—	—	—	—	—	<0.35	<0.35	7.68
OAS #5	—	—	—	—	—	—	—	—	—	<0.35	0.62	54.7

ISU, ISAC standard units; OAS, oral allergy syndrome; PA, peanut allergy.

TABLE E2. Source material for the estimation of the representation of the dominant allergens in the 5 extracts used for sIgE assays

Allergen extract	Specific allergen	Extract representation	Reference
Beef	α -gal	<1%	Mullins et al ^{E2}
Peanut	Ara h 2	9%	de Jong et al ^{E3}
	Ara h 8	1%-5%	Hebling et al ^{E4}
<i>Dermatophagoides pteronyssinus</i> (mite)	Der p 1	30%-70%	Grier et al ^{E5}
	Der p 2	30%-70%	
Cat dander	Fel d 1	>80%	Grier et al ^{E5}
Cow's milk	Bos d 4	5%	
	Bos d 5	10%	
	Bos d 6	1%	Wal ^{E6}
	Bos d 8	80%	
	α -gal	<1%	Mullins et al ^{E2}

TABLE E3. Titer of specific IgG₄ to and IgG₄:IgE ratios for dominant cow's milk proteins in sera of 10 out of 11 patients with EoE who were positive for sIgE to cow's milk extract (there was not sufficient serum for IgG₄ assays for EoE #10)

Patient	Percentage difference	Dominant allergen	Specific IgG ₄ to dominant allergen (μ g/mL)	IgG ₄ :IgE ratio for dominant allergen
EoE #5	+47%	Bos d 6	0.28	8.8
EoE #6	+3%	Bos d 8	3.83	232.1
EoE #9	+285%	Bos d 6	18.0	4250
EoE #11	+296%	Bos d 4	64.4	2940
EoE #12	+7%	Bos d 5	3.04	184.7
EoE #13	+22%	Bos d 5	72.3	1966
EoE #14	+87%	Bos d 4	6.44	187.4
EoE #15	+209%	Bos d 6	8.15	80.2
EoE #16	+15%	Bos d 8	0.64	10.9
EoE #17	+30%	Bos d 4	6.09	82.5

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