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Egner, W., Swallow, K., Lock, R.J. et al. (1 more author) (2016) Falsely low IgG4 in routine analysis - How not to miss IgG4 disease. Clinical & Experimental Immunology, 186 (1). pp. 57-63. ISSN 0009-9104

https://doi.org/10.1111/cei.12805

Title: Falsely low IgG4 in routine analysis – How not to miss IgG4 disease.

Short title: IgG4 prozoning

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Key words: IgG4, IgG subclasses, Prozoning

Abbreviations: IgG4 related disease (IgG4-RD), United Kingdom National External Quality Assurance Scheme (UK NEQAS), United Kingdom National External Quality Assurance Scheme Immunology, Immunochemistry and Allergy (UK NEQAS IIA), Method laboratory trimmed mean (MLTM), Standard deviation (SD)

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/cei.12805

Abstract

Background

IgG4 disease can have apparently "normal" levels of IgG4 due to antigen excess conditions. IgG4 measurement therefore appears falsely low. UK NEQAS data and other reports have suggested this problem occurred despite pre-existing antigen excess detection steps.

Methods

We examined the prevalence and characteristics of prozoning in our laboratory and patient cohorts, to determine the clinical relevance of the problem.

Results

We establish that the prevalence of raised IgG4 in routine IgG4 analysis is low (<1%) using one of the 2 routine methods in use in the UK. We show that subsequent assay modification appears to have reduced the likelihood of misleading readings. However, the original version of the assay prozoned to low levels (below 0.64g/L) in 41% of high IgG4 samples in our patients. This may explain the previous reports of low sensitivity of raised IgG4 for IgG4RD, and predictive values should be re-evaluated in this disease using modified prozone-resistant protocols.

Conclusions

All laboratories providing IgG4 measurements should verify that their assays are fit for the clinical quality requirement of detection raised IgG4 levels and must verify the upper limit of their reference ranges and freedom from prozoning.

Introduction

Measurement of the IgG4 Immunoglobulin subclass was thought to be of limited utility until the recent description of IgG4 related disease (IgG4-RD) (1-15). A cardinal criterion of IgG4 disease, unsurprisingly, is a raised IgG4 level. However most clinicians do not understand that IgG4 measurement is more complex than other isotypes. IgG4 is an unusual form of immunoglobulin with unique properties. There are at least 2 different assays, producing different values, with different reference ranges. Furthermore, UK National External Quality Assessment Schemes (UK NEQAS), and other publications, have demonstrated the existence of antigen excess phenomena at moderate levels of IgG4, not much above the 95th centile of the reference range, sufficient to cause the raised IgG4 to be missed due to prozoning (4, 16, 17).

Historically, IgG4 was measured alongside the other IgG subclasses (IgG1, IgG2 and IgG3) in the investigation of immune deficiency (18-20). Hence assays were optimised to detect low or normal levels of IgG4. The Binding Site IgG4 assay had a limited detection level above the normal range (upper detection limit 2.4g/L). However all IgG4 assays were also unable to measure the lower limit (21). Therefore quoted reference ranges have lower limits of "zero". The use of IgG4 measurement to investigate IgG4-RD has now changed the measurement range of clinical relevance to include raised levels.

Antigen excess, or prozoning, occurs in fluid phase immunoassays due to the Heidelberger-Kendall kinetics curve which shows the altered interaction between antigen and antibody at varying concentrations (22, 23). In

nephelometric/turbidimetric systems, the same output signal, can be produced by two samples of differing antigen concentration, one in antibody excess the other in antigen excess. One measurement is correct the other is falsely low.

In antigen excess conditions the antigen competes for binding sites on the antibody leading to resolubilisation of immune complexes and a reduction in signal detected (Figure 1). Many analysers have a mechanism to detect if the sample might be in antigen excess and a further dilution is performed to create antibody excess conditions for analysis. If this check is absent, or fails, the result generated may be falsely low (Figure 1).

In December 2012, UK NEQAS Immunology, Immunochemistry and Allergy (UK NEQAS IIA) suggested that some participants in the IgG subclasses scheme should investigate the possibility of antigen excess, because some laboratories submitted results in the normal range when the consensus for that method gave an elevated value for IgG4 (Sample 126-2, Method laboratory trimmed mean (MLTM) target 3.7g/L). This phenomenon was reproducible (samples 132-2 and 136-2) and affected laboratories randomly, with most laboratories detecting samples on one occasion and not others.

In 2014, The Binding Site issued an article (21) detailing changes to their IgG4 assay protocol to incorporate a two-step antigen excess check, which was aimed to resolve the problem. These changes are specific to The Binding Site IgG4 assay on the Siemens BNII analyser. The original antigen excess check protocol added the total volume of patient sample required to the reaction

mixture in one step. In the new protocol, a small amount of sample is added to the reaction mixture initially and then a second aliquot is added following a short interval. This allows any changes in reaction rates to be observed and determine if the sample might be in antigen excess and the result unreliable.

This study had two main aims:

1. To investigate antigen excess in the UK NEQAS IIA sample 126-2.
2. To perform a series of studies to determine the prevalence of prozoning in routine use and to check that the new two-step antigen excess check resolved the issue. We tested samples from patients with a range of clinical indications including immune deficiency and IgG4-RD.

Materials and Methods

Samples were analysed on the Siemens BNII nephelometer (Siemens, Camberley, Surrey, UK) using The Binding Site (Birmingham, UK) IgG4 reagent according to the manufacturer's instructions. All samples were initially tested at a starting dilution of 1/100 on the BNII unless otherwise stated. Samples were tested at further dilutions if automatically triggered when the sample appeared to exceed 0.64g/L. The laboratory performs three-level internal quality control prior to each run (low: 0.09g/L, CV= 8.05%; Medium: 0.28g/L, CV= 4.53%; High: 0.49g/L, CV= 5.19%) and participates in the UK NEQAS IIA scheme for IgG subclasses with good performance.

UK NEQAS IIA Sample 126-2 and Patient Linearity

Aliquots of sample 126-2 were used for linearity experiments.

Linearity was also checked on two samples (S1 and S2), both apparently within the adult reference range of <1.3g/L (S1 at 0.71g/L, S2 at 1.19g/L) and one known to be in antigen excess (AgXS 1: initial result 0.27g/L but 4.6g/L with further dilution). The samples were analysed neat and at serial dilutions in phosphate buffered saline. All samples were analysed using the original IgG4 protocol with a one-step antigen excess check at an on-board dilution of 1/100. The results were plotted to determine linearity of the measurements for each sample.

The frequency of hidden prozoning in patients with apparently low normal IgG4 levels (<0.64g/L)

We retrospectively re-tested 122 random samples at 1/400 dilution. All were originally reported with IgG4 in the range 0.05-0.64g/L (below the range which would trigger an antigen excess check by 1/400 auto-dilution).

Frequency of Prozoning in routine sample measurement using both protocols

199 unselected, consecutive samples requesting IgG subclasses (February 2015 to March 2015) were tested using both the original one-step antigen excess protocol and the improved two-step antigen excess protocol.

Prospective evaluation of new antigen excess detection protocol

2066 consecutive, unselected samples were measured with the new protocol (June 2014 – February 2015). Since the manufacturers had proposed that only samples with results >2.4g/L would have prozoned (21), we elected to

test all samples above 1.8g/L (i.e. 2.4g/L minus 2 standard deviation (SD) in our assay) to ensure that all samples around the 2.4g/L cut-off were included. 41/2066 samples (2%) were >1.8g/L and were re-checked on the original protocol. We defined a definitively prozoning sample as one which changed >2SD after antigen excess check, as this is the threshold at which we have a 95% probability of a true difference in values.

Retrospective analysis of IgG4 results: June 2011 – February 2015

Since a change in the method protocol had occurred in June 2014, we needed to verify that the reference range had not changed (June 2011-February 2015). The results were compared using Analyse-it software version 2.3 (Leeds, UK).

Results

UK NEQAS IIA sample 126-2 and linearity

The original protocol appears linear up to 2.4g/L.

Prozoning in UK NEQAS EQA distributions was a frequent occurrence (21 of 63 measurements, 33%) in laboratories utilising affected method (Table 1). It occurred in different laboratories each time. One laboratory prozoned on all 3 distributions and 1 prozoned on 2 distributions. The remaining 19 laboratories only prozoned on one occasion.

In our laboratory antigen excess effects were again seen in 5 of 6 aliquots of the same UK NEQAS IIA sample that originally demonstrated the effect (126-2) with the original antigen excess protocol. Confirming that earlier prozone

detection was variable and not robust within a single laboratory. One of 6 replicates obtained the correct value of 4.0g/L consistent with the assigned value. The remaining 5 aliquots gave severely suppressed results between 0.58-0.64g/L. Samples >2.4g/L do not always trigger automatic dilution as the original protocol was not set up to detect raised levels (21). Subtle variability in the IgG4 levels measured in these aliquots may account for one sample being able to automatically trigger further dilutions by giving a value just above the dilution threshold, whereas the majority of the aliquots did not do this.

AgXS 1 was linear up to 2.3g/L and then prozoned when analysed in the usual way, similar to the pattern seen in the UK NEQAS IIA sample 126-2 (Figure 2). These results confirm the manufacturer's data on the upper limit for the original antigen excess check (21). Samples known to have results below 2.3g/L (S1 and S2) had linear recovery across all the dilutions tested (Figure 2).

The frequency of hidden Prozoning in low IgG4 levels (<0.64g/L)

4 of 122 randomly selected "low IgG4" samples (3.3%) were in antigen excess and actually had raised IgG4 (Table 2). Samples 1 and 2 are so close to the 0.64g/L threshold used to trigger automatic re-dilution that they may have been missed on some occasions due to normal assay variation (CV 5-10%).

Like many laboratories, we do not routinely test total IgG on all requests for IgG subclasses, and they would mostly be done together when immunodeficiency was suspected. Therefore the difference between the sum of the individual IgG subclasses and total IgG was not available for

comparison. It is doubtful that this is a reliable check unless the IgG4 is very high, since total IgG assays may have a CV of ≤10% and thus cannot reliably detect discrepancies of +/-2g/L at a 10g/L level.

Frequency of Prozoning in routine sample measurement using both protocols

We included samples with detectable and undetectable (<0.05g/L) IgG4. The samples with undetectable levels were included in case any prozoning sample resulted in an apparently unmeasurable signal.

Only 1 of the 199 consecutive samples tested prozoned using the old protocol (from 6.8g/L to 0.2g/L). 7 samples were <0.05g/L, 143 samples 0.05-0.64g/L (1/100 measuring range), 47 samples 0.65 -2.4g/L (1/400 measuring range) and 2 gave results >2.4g/L (2.41g/L and 2.47g/L). This suggests a prevalence of hidden antigen excess of only 0.5%.

Prospective evaluation of new antigen excess detection protocol in 2066 consecutive samples

The estimated prevalence of antigen excess was 0.8% (17/2066) when using the new protocol and re-running samples above 1.8g/L on the old protocol (Table 3). This confirms the estimated prevalence of 0.5% in 199 consecutive samples.

14 of these samples would also have been identified by a 'belt and braces' check protocol of repeat testing of samples with an initial result below 0.64g/L on the original protocol (samples 1-14) (Table 3).

Comparison of old and new antigen excess check performance on the same samples

In total, 240 samples have been tested using both protocols to allow a direct comparison of results. A Passing-Bablock comparison plot (Figure 3) highlights that the majority of cases of antigen excess had a misleading result on the original protocol of <0.64g/L.

Non-prozoning results up to an IgG4 of 2.5g/L are comparable using both protocols (but obviously does not exclude the possibility that true antigen excess could rarely produce a result in this range in some samples).

Retrospective analysis of IgG4 results: June 2011 – February 2015

The normal range for adults (0 – 1.3g/L) was checked using data produced on the new protocol from June 2014 – February 2015 (Reference interval confirmation using Analyse-it v2.3, Anderson-Darling A^2 non-parametric normality test). We confirmed that there was no significant change to the values obtained using both protocols in samples that did not prozone (Pearson correlation coefficient r = 0.98, Analyse it v2.3).

Figure 4 indicates that although we can now detect IgG4 more reliably, there frequency of high IgG4 levels remains low in routine laboratory practice. The percentage of samples giving results >1.3g/L over the study duration has not changed significantly: June 11-Feb12 = 4.2%, June 12-Feb13 = 4.9%, June 13-Feb14 = 4.7% and June 14-Feb15 = 4.2%.

Discussion

The Binding Site IgG4 assay on the Siemens BNII analyser originally claimed an antigen excess check capability of 2.4g/L (21) It is now apparent that samples with IgG4 greater than 2.4g/L could potentially go undetected and result in the prozoning phenomenon as the assay was not configured to detect IgG4 disease with raised levels originally. New disorders are described frequently and may change the clinical use of established assays. Assay verification or validation needs to include assessment of performance against clinically derived acceptable performance characteristics (the clinical quality requirement). The recent recognition of IgG4 disease and diagnostic use of with raised IgG4 levels is an exemplar of this phenomenon. Previous assay verification and development strategies may not have been targeted at this performance characteristic.

Recently, The Binding Site have introduced an amended assay protocol with a two-step antigen excess check to increase the reliable measuring range of the assay to 49g/L (21) which appears to have successfully mitigated the problem. Users of other IgG4 assays should now also verify that prozoning is not an issue with their methods.

It has been claimed that up to a quarter of raised IgG4 levels may exhibit significant prozoning in selected series (16). This could mean that cases of possible IgG4 disease are being missed, however it is important to note that IgG4-RD can present with normal or low IgG4 levels and current diagnostic guidelines include other criteria such as biopsy for this reason (6, 9-11). The

prevalence of prozoning described here is much lower than that stated by Khosroshahi et al, because we are establishing the prevalence in a random cohort of patients, most of whom are not suspected of having IgG4 related disease. However our evaluation of our selected raised IgG4 samples suggests that the prevalence of prozoning in this cohort may be even higher at >40%. This would be a serious problem with detecting a rare disease and could explain the apparent lack of raised IgG4 in some “IgG4 Disease” cases (14-17). In our cohort the prevalence of significantly prozoning samples was <1% of routine sampling, but 4 in 10 of those might have been previously missed.

The positive predictive value of the test is reportedly <40% for IgG4-RD prior to effective antigen excess detection, but this may be an artefact of both test and cohort biases and is not generalizable. It was established using a cut-off of 1.35 and both Siemens and This Binding Site IgG4 reagent. (17).

Diagnostic guidelines for autoimmune pancreatitis (9) and IgG4 related disease IgG4-RD (6) both utilise IgG4 measurement when investigating these conditions. Elevated IgG4 (> upper limit of the reference range or 1.35g/L) is suggestive of these diagnoses in the correct clinical context, but that raised results can be observed in other conditions and that IgG4 disease may still be present if the IgG4 is normal (24). Caution is required when clinicians interpret these results as it is clear that the upper limit of the reference range is different for the 2 main assays used (Binding site 0.039 – 0.864g/L and Siemens 0.03 – 2.01g/L) due to the lack of standardisation of calibration (25). If 1.35g/L is arbitrarily used, it falls within the “normal range” for the Siemens

assay. Laboratories should highlight their locally verified upper limits of normality on their reports.

The Binding Site has now modified its assay successfully to ameliorate the problem (21). A new protocol employing a two-step check has been created and details are now supplied with the reagents, but users must choose to implement this change and it is not currently mandatory. It should be implemented in all laboratories measuring IgG4.

We recommend that all users of this assay check their protocols, implement the use of effective antigen excess checks if they have not already done so, and verify both the upper limit of normal and the linear working range of their assay to ensure they are fit for purpose, in line with laboratory ISO accreditation requirements. Adjustment of the reference range was not required in our system.

Any immunoassay that uses antibody-antigen interactions are at risk of similar phenomena if high levels of analytes are of clinical importance. Review of the clinical use of the assay in formulating a clinical quality requirement should highlight those assays where extra verification of prozoning or interferences is required. It is also important to consider antigen excess in other assay formats [22].

Disclosures

The authors declare no conflicts of interest.

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Figure legends:

Figure 1: Heidelberger Kendall curve. There are 3 zones: antibody excess, equivalence and antigen excess. A sample in antigen excess can give the same result as one in antibody excess – as indicated by the grey arrow. Assays are designed to produce most results in antibody excess conditions to prevent falsely low results being reported.

Figure 2: Linearity experiment results. Shows that all samples are linear below 2.3g/L. 126-2 and AgXS1 were non-linear above this level and both dropped close to the 0.64g/L threshold that would trigger an antigen excess check. This may explain why some laboratories detected it on one occasion but not another.

Figure 3: Scatter plot with Passing and Bablock fit in 240 samples tested on both protocols. All those which show inconsistent results were below 0.64g/L on the original protocol and greater than 2.4g/L on the new protocol.

Figure 4: High IgG4 results are detected more reliably since the change in protocol (Feb 2014). Upper linear limit of measurement in the original protocol (2.4g/L) indicated by the dashed line. Results indicate a large increase in values and detection of raised IgG4, without any known change of testing patterns or increase of requesting.

UK NEQAS IIA distribution	MLTM* target (g/L)	CV %	Total responses	No. Prozoning samples (within adult reference range)	% Prozoning Samples
126 (sample 126-2)	3.7	22	19	4	21%
132 (sample 132-2)	3.1	61	22	10	46%
136 (sample 136-2)	3.9	30	22	7	32%

Table 1: UK NEQAS IIA Distributions with High IgG4 levels**.** Results form users of The Binding Site assay showed that prozoning frequency varied between distributions from 21 – 46%. This also led to large assay %CV for these samples. *MLTM = Method Laboratory Trimmed Mean.

Sample	IgG4 at 1/100 dilution (g/L)	IgG4 at 1/400 dilution (g/L)
1	0.63	4.62
2	0.57	5.13
3	0.27	6.05
4	0.15	12.5

Table 2: Four prozoning samples which originally did not trigger auto dilution because they appeared to be <0.64g/L – the threshold which triggered an antigen excess check on the original protocol.

Sample	IgG4 (g/L) on original protocol	IgG4 (g/L) on new protocol	Evidence of prozoning?
1	0.18	12.1	Yes
2	0.22	12.3	Yes
3	0.23	2.78	Yes
4	0.24	9.51	Yes
5	0.28	5.69	Yes
6	0.28	11.4	Yes
7	0.31	2.57	Yes
8	0.31	6.09	Yes
9	0.36	3.79	Yes
10	0.38	3.89	Yes
11	0.41	4.62	Yes
12	0.42	3.41	Yes
13	0.46	3.53	Yes
14	0.55	4.47	Yes
15	1.39	2.08	Yes
16	1.57	2.07	Yes
17	1.72	1.85	No
18	1.8	2.03	No
19	1.8	1.9	No
20	1.85	1.86	No
21	1.87	2.06	No
22	1.9	2	No
23	1.92	1.93	No
24	1.92	2.06	No
25	2.01	2.21	No
26	2.09	1.87	No
27	2.1	2.07	No
28	2.1	2.17	No
29	2.14	2.57	No
30	2.15	1.99	No
31	2.18	2.14	No
32	2.23	2.57	No
33	2.26	2	No
34	2.27	1.95	No
35	2.33	3.37	Yes
36	2.39	2.32	No
37	2.48	2.41	No
38	2.57	2.81	No
39	2.61	2.82	No
40	2.78	2.69	No
41	2.94	2.51	No

Table 3: Assay modification detects samples >1.8g/L better than the original assay. 41% of samples>1.8g/L on the new protocol showed prozoning. 100% of samples prozoning were over 2.57g/L.