



Measurement of Specific IgG Anti-Fel d 1 Antibodies

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Abstract

There is currently considerable interest in the role of specific IgG antibodies in allergy. Several studies suggest that specific IgG antibodies may play a protective role in allergy. Successful immunotherapy is associated with increases in allergen-specific IgG antibodies which correlate with clinical outcome. Other studies have identified an inverse relationship between exposure to cat and sensitization, which was associated with high-titer-specific IgG and IgG₄. This immune response was described as a modified Th2 response, since both IgE and IgG₄ require Th2 cytokine IL-4 for their production. A modified Th2 response was described with laboratory animal allergy, where there was almost a twofold reduction in the risk of developing work-related chest symptoms.

In this chapter, we review the major factors to be considered in the development of an ELISA for the determination of specific IgG and IgG₄ antibodies.

Key words IgG antibodies, IgG₄ antibodies, IgE, modified Th2 response, Blocking antibodies

1 Introduction

There has been considerable interest in the role of IgG in allergy: recent evidence suggests that it may play a protective role. An inverse relationship between exposure to cats and sensitization was described which was associated with high-titer-specific IgG and IgG₄ [1]. This immune response is described as a modified Th2 response, since both IgE and IgG₄ require Th2 cytokine IL-4 for their production. The modified Th2 response is suggestive of clinical tolerance since the shift from specific IgE antibodies to IgG₄ resulted in a decrease in both sensitization and asthma [2].

We have recently established a modified Th2 response in laboratory animal workers. Within our cohort and cross-sectional studies on laboratory animal allergy we observed increasing risks of sensitization and work-related symptoms with increasing exposures to rats, except at highest exposure level where risks of both outcomes were lower [3, 4]. We established a significantly increased ratio of IgG₄:IgE in those workers most heavily exposed. There was

an almost twofold reduction of symptoms in those who produced both specific IgG₄ and IgE as compared to those producing specific IgE only. The attenuation of sensitization and symptoms at high exposures and the increased ratio of IgG₄:IgE in laboratory animal workers are suggestive of a natural form of immunotherapy.

Ratios of IgE to IgG₄ have been found to change during specific immunotherapy [5]. Specific serum levels of both IgE and IgG₄ increase during the early phase of therapy, but the increase in specific IgG₄ is more pronounced and the ratio of specific IgG₄:IgE is increased 10- to 100-fold, suggesting a protective role for IgG₄ [5]. IL-10 is a potent suppressor of both total and allergen-specific IgE, whereas it simultaneously increases IgG₄ production [6].

Successful immunotherapy is associated with increases in allergen-specific IgG₁ and IgG₄ antibody concentrations occur that correlate with the clinical outcome [7–10]. It is not known whether allergen-specific IgG or IgG₄ antibodies associated with the modified Th2 response exhibit functional activity. It is possible that IgG antibodies may play a protective role by blocking leukocyte histamine release, inhibiting signal transduction and mediator release through the high-affinity IgE receptor (FcεR1) and IgG (FcγRIIB) receptors [11–14] and blocking allergen-induced IgE-dependent histamine release by basophils [15]. IgG antibodies may exert their effect by competitive inhibition of allergen-IgE complexes, which prevents complexes binding to the low-affinity IgE receptor, CD23, and subsequent antigen presentation. Others have suggested that not only quantitative changes occur with the IgG antibody but also the spectrum of the specificity of the IgG is altered [16]. One grass pollen immunotherapy study demonstrated a blunting of seasonal increases in serum allergen-specific IgG and IgG₄. Further examination showed the postimmunotherapy serum to exhibit inhibitory activity, which co-eluted with IgG₄ and blocked IgE-facilitated binding of allergen-IgE complexes to B cells. Increases in IgG and the IgG blocking activity correlated with the patients' overall assessment of improvement [9].

2 Materials

2.1 ELISA

1. Elisa plate.
2. 1× Phosphate-buffered solution (PBS).
3. Allergen at 1 µg/mL Fel d 1 in PBS.
4. Positive control: A pool of serum samples known to have IgG anti-Fel d 1 antibodies.
5. Negative control: A pool of serum samples known to be negative for IgG anti-Fel d 1 antibodies.

6. Test serum samples for the measurement of IgG anti-Fel d 1 antibodies.
7. Coating buffer: 50 mM Carbonate bicarbonate buffer pH 9.6.
8. Blocking buffer: PBS, 1% bovine serum albumin (BSA). Make up fresh on the day of assay.
9. Reagent buffer: PBS, 1% bovine serum albumin (BSA), and 0.05% Tween 20. Make up fresh on the day of assay.
10. Wash buffer: PBS, 0.05% Tween 20. Add sodium azide 0.05% for storage.
11. Biotinylated anti-IgG antibody (Becton Dickinson, Cowley, UK) diluted 1:1000 in reagent buffer: 10 mL is sufficient for 96-well ELISA plate.
12. ExtrAvidin Peroxidase diluted 1:1000 in reagent buffer.
13. Phosphate citrate buffer pH 5.0: 2.48 g Na₂HPO₄ to 50 mL water—buffer A. 1.92 g citric acid to 50 mL water—buffer B. Add 50 mL of buffer A and B to 100 mL deionized water and bring pH to 5.0. Store at 4 °C.
14. Stock substrate: Dissolve 100 mg 3,3',5,5'-tetramethylbenzidine (TMB) in 10 mL dimethyl sulfoxide. Store in 200 µL aliquots at −20 °C.
15. Substrate solution: Add 100 µL stock TMB stock solution to 10 mL phosphate citrate pH 5.0 and add 3 µL H₂O₂ (w/w) immediately before use. The substrate solution must be made prior to addition to wells. 10 mL Substrate solution is sufficient for one 96-well ELISA plate.
16. Stop solution: 1.9 M Sulfuric acid. Store at room temperature. N.B.: Always add acid to water in fume cupboard.

2.2 Inhibition Assay

1. In addition to the reagents for the ELISA, a higher concentration of Fel d 1 at 10 µg/mL is required for preincubation with serum.

3 Methods

The method described for the measurement of IgG antibodies specific for Fel d 1 has been optimized. A checkerboard was set up with varying concentrations of allergen, biotinylated antibody, and streptavidin peroxidase (*see Note 1*). The optimum concentrations were used for the ELISA. The time and temperature of the incubation periods were also optimized.

We believe that it is essential to subtract the binding of IgG antibodies to the blocking agent BSA. Some sera have much higher binding to BSA than other sera; however it is essential to subtract the background binding for each individual.

We have measured specific IgG antibodies to a panel of allergens. The protocol given for the measurement of IgG-specific antibodies to Fel d 1 can be optimized for each allergen using the checkerboard approach. IgG₄-specific antibodies can also be measured using the same protocol but substituting the biotinylated anti-IgG antibody with biotinylated anti-IgG₄ antibody. The IgG₄ ELISA will need to be optimized as above.

**3.1 Protocol
for IgG-Specific
Antibodies to Fel d 1**

1. Coat each well in the upper half of the ELISA plate with 100 μ L of 1 μ g/mL Fel d 1.
2. Coat each well in the lower half of the ELISA plate with 100 μ L of 1% BSA.
3. Store in a polystyrene box with a damp tissue overnight at 4 °C.
4. Wash plates $\times 3$ with wash buffer. Plates can be washed by hand using a squeeze bottle or using a dedicated ELISA washer (*see Note 2*). The hand method is suitable for a small number of plates. At the end of the third wash bang the plate on a tissue to get most of the remaining buffer out of the wells.
5. Add 200 μ L of blocking buffer to each well in the whole plate. Incubate in a damp box for 2 h at 4 °C.
6. Wash plates $\times 4$ with wash buffer.
7. Gently mix serum samples on vortex. Add 100 μ L of reagent buffer to wells A1–2 and E1–2. Add 10 μ L of positive control and 90 μ L reagent buffer to wells A3–4 and E3–4. Add 10 μ L negative control and 90 μ L reagent buffer to wells A5–6 and E5–6. Add 10 μ L of test serum to wells in duplicate to both the upper allergen-coated plate and the BSA-coated plate. Incubate for 2 h at 4 °C.
8. Wash plates $\times 5$ with wash buffer.
9. Add 100 μ L of biotinylated anti-human IgG (diluted 1:1000 in reagent buffer) to each well and incubate for 1 h at 4 °C.
10. Wash plates $\times 6$ with wash buffer.
11. Add 100 μ L of ExtrAvidin Peroxidase (diluted 1:1000 in reagent buffer) to each well and incubate for 30 min at 4 °C.
12. Wash plates $\times 7$ with wash buffer.
13. Add 100 μ L of freshly made substrate solution to each well. Leave for 10 min at room temperature.
14. Add 50 μ L of stop solution to each well.
15. Wipe the bottom of ELISA plate with a tissue and ensure that there are no air bubbles in any of the wells. Read ELISA plate at 450 nm absorbance.
16. Calculate the average of duplicates.

3.2 Protocol for Determining Specificity of ELISA by Inhibition Assay

17. Subtract the background BSA OD measurement from the allergen OD measurement for each sample. The results are arbitrary values based on OD values.
1. Coat each well in the upper half of the ELISA plate with 100 μ L of 1 μ g/mL Fel.
 2. Coat each well in the lower half of the ELISA plate with 100 μ L of 1% BSA.
 3. Store in a polystyrene box with a damp tissue overnight at 4° C.
 4. Incubate serum with varying quantities of allergen to set up an inhibition curve. In our Fel d 1 assay we incubated equal volume of serum sample with Fel d 1 solution at 1.0, 0.5, 0.1, and 0 μ g/mL final concentration in the well. The serum and allergen were left on a roller at 4 °C overnight.
 5. Wash plates $\times$ 3 with wash buffer.
 6. Add 200 μ L of blocking buffer to each well in the whole plate. Incubate in a damp box for 2 h at 4 °C.
 7. Wash plates $\times$ 4 with wash buffer.
 8. Add 10 μ L of the preincubated serum and allergen to the wells and incubate for 2 h at 4 °C.
 9. Continue with ELISA as for above protocol from **step 8** onwards.
 10. The degree of allergen inhibition is taken as (uninhibited – inhibited)/inhibited $\times$ 100. An example of our inhibition curve for Fel d 1 is shown in Tables 1 and 2 for two different sera.

3.3 Analysis of ELISA Data

We did not have the appropriate reagents to set up a standard curve for our Fel d 1 assay; we therefore reported our results as arbitrary values based on the optical density. We had to ensure that samples run in different assays were compatible, so we ran a pool of serum

Table 1
Inhibition of IgG anti-Fel d 1 antibodies binding with the solid-phase Fel d 1 with inhibitor Fel d 1 allergen

Inhibitor μ g/ mL	O.D. Feld 1	O.D. HSA	O.D. Fel d 1—HSA	% Inhibition
0	0.947	0.430	0.517	0
0.1	0.761	0.388	0.373	27.8
0.5	0.740	0.369	0.371	28.3
1.0	0.521	0.445	0.076	85.3

Table 2
Inhibition of IgG anti-Fel d 1 antibodies with the solid-phase Fel d 1 with inhibitor Fel d 1 allergen

Inhibitor $\mu\text{g}/\text{mL}$	O.D. Fel d 1	O.D. HSA	O.D. Fel d 1—HSA	% Inhibition
0	1.073	0.425	0.648	0
0.1	0.980	0.453	0.527	18.6
0.5	0.849	0.424	0.425	34.4
1.0	0.722	0.789	0	100.0

We were able to get 85–100% inhibition by preincubating two different sera with 1 $\mu\text{g}/\text{mL}$ Fel d 1. Less than 1 $\mu\text{g}/\text{mL}$ of inhibitor Fel d 1 did not significantly inhibit the binding of specific IgG anti-Fel d 1 antibodies to the solid phase. This demonstrated that the binding to Fel d 1 in the assay was specific to Fel d 1 and was not as a result of nonspecific or low-affinity antibody binding

with low, medium, and high levels of IgG antibody on each plate in every assay run. We ran these samples in ten different assays to establish the coefficient of variance. Our assays would only be accepted if the coefficient of variance of the quality control samples was less than 20%.

4 Notes

1. Optimization of assay.

Every step within the ELISA needs to be optimized [17]. In particular the following were considered when setting up the IgG assay.

- (a) Concentration of allergen on solid phase—the capacity of microtiter plates to bind proteins is limited. At high concentration of protein, there is a tendency for protein molecules to bind to each other, resulting in dissociation of bound protein during the assay. In practice 1–10 $\mu\text{g}/\text{mL}$ is the desired concentration to use for coating your solid-phase ELISA plate. A steep reference curve is desirable since a small change in concentration will give a large color change. In practice we would aim for a 2–3 log range in our dilution curves.
- (b) Choice of coating buffer—the choice of coating buffer should be determined by testing the binding of allergen with pH 9.6 carbonate/bicarbonate buffer, a pH 7.4 phosphate-buffered saline, and a pH 5.0 acetate/citrate buffer. The charge of a protein depends on the buffer it is dissolved in. Therefore the charge of the coating buffer

plays an important role in the ability of the allergen to bind to the ELISA plate.

- (c) Incubation time for coating plate—1 h is usually sufficient. However it is often desirable to coat the ELISA plate overnight, to allow time to perform the assay the following day. Again it is prudent to optimize the incubation time. It is possible to coat and block ELISA plates and keep frozen until ready for use. Again it is essential to check that this works for your particular assay.
- (d) Blocking plates—several blocking reagents can be used ranging among human serum albumin, bovine serum albumin, casein, and gelatine. Again when setting up your assay it is worthwhile comparing the various blocking agents. Typically the blocking agents are used at a concentration of 1–2%.
- (e) Volume of reagents—typically we use 100 μL of reagents per well, apart from in the blocking stage where we typically use 200 μL . If the sensitivity of the assay needs to be increased we use 200 μL reagent per well. Anything over 200 μL per well has the potential for crossover of reagent between wells.
- (f) Choice of buffer—the concentration and molarity of buffers are generally between 0.05 and 0.1 M. Antibody-antigen reactions normally take place between pH 6.0 and 9.0. Normally the buffers used in the assay will contain blocking buffer. It is essential that the buffers are made fresh on the day since addition of serum albumin can lead to bacterial contamination which will result in erroneous ELISA results.
- (g) Incubation times—1 h is usually sufficient for the reaction to be complete. The incubation times can be shortened by placing the ELISA plate on a shaker—the times can typically be cut in half. Again the incubation times will need to be optimized for each assay.
- (h) The dilution of the sample will depend on the concentration; low concentration will require high sample volume, whereas high concentration will need to be diluted. At high serum concentrations there is a tendency to get nonspecific binding and binding of low-affinity antibodies. The serum sample needs to be optimized by setting up a dilution curve.
- (i) Detecting antibodies—As with other steps in the ELISA these will need to be optimized. It is essential to ensure that the detecting reagent binds only to the sample and not to the other reagents in the ELISA.

2. We normally use a wash bottle to wash our ELISA plate which is perfectly adequate. This is a very simple effective cheap method of washing ELISA plates. It is essential for the user to be very thorough in their washing steps, ensuring that all wells are washed and that the buffer is flicked out of the plate. Some prefer to use an automated plate washer which is useful if running several plates in one assay. The downside of an automated plate washer is the expense; however it is useful if you intend running ELISAs on a regular basis and overcomes user error in the washing process. Others use a bucket of buffer and simply immerse the plate in the bucket of buffer which is a perfectly adequate system of washing.

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