



Measurement of Specific IgG₄ Anti-mouse Urine Antibodies

Jennifer Canizales

Abstract

IgG₄ and its role in immune tolerance has been investigated widely. Symptom reduction and improved clinical outcomes in immunotherapy trials are associated with significant increases in allergen-specific IgG₄ antibodies. Natural immune tolerance observed in beekeepers and cat owners has also been associated with elevated levels of bee venom and cat allergen-specific IgG₄, respectively. Functionally, allergen-specific IgG₄ has been shown to reduce the binding of IgE-allergen complexes to B cells, a key step in the initiation of the type 1 hypersensitivity allergic response. In laboratory animal allergy, IgG₄ has been described as a “protective/blocking” antibody. However, a consensus on the exact relationship between exposure, IgG₄, and tolerance has yet to be reached. In this chapter, we review the factors that require consideration when developing an ELISA for the quantification of allergen-specific IgG₄.

Key words IgG₄ antibodies, Blocking antibodies, Modified Th2 response, Natural immune tolerance

1 Introduction

Allergy, the type 1 hypersensitivity reaction, is mediated by IgE antibodies. Sensitization first occurs when an individual is exposed to an allergen and their immune cells (B cells) produce specific IgE antibodies to this allergen. These IgE antibodies opsonize/coat immune cells such as mast and basophil cells. Upon re-exposure the allergen can be bound by the specific IgE on the surface of these cells causing “cross-linking,” which leads to degranulation of the mast cells and release of histamine and other pro-inflammatory mediators, leading to the common allergic symptoms such as rhinitis.

Those exposed to allergens can also develop allergen-specific IgG and IgG₄ antibodies. There is much interest in these antibodies as they have been implicated in the anti-inflammatory T-regulatory response [1], as well as being shown to block the binding of allergen-IgE complex binding to B cells via the FcεRII receptor [2, 3]. Both IgE and IgG₄ are produced as part of the Th2 response [4]. Cat allergy studies have shown an association between high cat allergen exposures, production of high levels of

allergen-specific IgG₄ (without specific IgE), and clinical tolerance. This suggested switch from the production of specific IgE to specific IgG₄ is known as the “modified Th2 response” [5, 6].

The role of IgG₄ antibodies in tolerance has been widely studied in relation to allergy immunotherapy. It has been established that the administration of allergen immunotherapy leads to a marked increase in the levels of allergen-specific IgG₄ antibody [7–9]. However, it is the level of blocking activity of the IgG₄ antibody (preventing the binding of IgE-allergen complexes to B cells) that directly correlates with immunotherapy treatment efficacy [10]. It may also stand to reason that allergen-specific IgG₄ antibodies also have a role in natural immunotolerance. Studies of beekeepers have demonstrated a positive association between chronic/high exposure (number of stings) and higher levels of bee venom-specific IgG₄, this suggests a role for specific IgG₄ antibodies and natural tolerance [11, 12].

Our work focuses on the role of IgG₄ in natural immunotolerance to laboratory animal allergy (LAA). Many studies have shown a positive correlation between increasing levels of rodent exposure and levels of rodent-specific IgG₄ [13–15]. However, a recent cohort study found no such correlation [16].

Reports vary on whether there is an association between specific IgG₄ and clinical symptoms. A number of studies have shown that increased levels of specific IgG and IgG₄ are positively associated with, or predictive of, sensitization and symptoms [17]. Others suggest that higher levels of specific IgG₄ in fact reduce the risk of symptoms and sensitization [15]. The importance of antibody ratios has also been investigated. Jeal et al. [13] found that increased rat-specific IgG₄ correlated with increased rates of symptoms and sensitization; however, when looking at the ratios of specific IgG₄ to specific IgE, this was reversed (high specific IgG₄/low specific IgE ratio = low symptoms and sensitization). Similarly to immunotherapy trials, the functional role of IgG₄ in LAA has been investigated. It has been shown that increased levels of rat-specific IgG₄ are associated with reduced levels of IgE-allergen complex binding to B cells [13].

2 Materials

2.1 ELISA

1. ELISA plate (Corning EIA/RIA Assay Microplate, Sigma-Aldrich, UK).
2. 1× Phosphate-buffered saline (PBS)
3. In-house mouse urine extract in PBS.
4. Positive control: Pool of serum samples from laboratory animal workers previously identified as having detectable levels of mouse urine-specific IgG₄.

5. Negative control: Pool of serum samples from non-laboratory animal workers, previously identified as being negative for mouse urine-specific IgG₄.
6. Test serum samples for the measurement of mouse urine-specific IgG₄.
7. Reagent/blocking buffer: PBS, 1% bovine serum albumin (BSA), 0.05% Tween-20. Make up fresh on the day of assay.
8. Wash buffer: PBS, 0.05% Tween-20. Make up fresh on the day of assay.
9. Natural Human IgG₄ Protein (Abcam, Cambridge, UK): Aliquot and store at -20°C .
10. HRP mouse anti-Human IgG₄ (Thermo Fisher Scientific, UK): Add glycerol for a final concentration of 50% and store at -20°C . To be diluted 1:1000 in reagent/blocking buffer, 12 mL is sufficient for 96-well ELISA plate.
11. Substrate solution: Pierce[™] TMB Substrate Kit (Thermo Fisher Scientific, UK): Substrate solution should be made up immediately before use. The two solutions provided in the kit should be mixed in equal parts; 12 mL substrate solution is sufficient for 96-well ELISA plate.
12. Stop solution: 2 M Sulfuric acid. Store at room temperature.
13. Ensure that there are no air bubbles in any of the wells and read plate at 450 nm absorbance.
14. Calculate average OD values of duplicates.
15. Using the average OD values determine the concentration of serum IgG₄ using the standard curve (described below), and express concentrations as arbitrary units (AU).

3 Methods

The method described for the measurement of IgG₄ antibodies specific for mouse urine and the standard curve for the ELISA has been optimized. A checkerboard was set up with varying concentrations of allergen, HRP-labeled antibody, and human IgG₄. The optimum concentrations were used for the ELISA. The time and temperature of the incubation periods were also optimized (*see Note 1*).

3.1 Protocol for Human IgG₄ Standard Curve

1. Make up human IgG₄ at a concentration of 125,000 pg/mL in PBS. This will be the highest/first point of the standard curve.
2. From the 125,00 pg/mL human IgG₄, make 11 serial dilutions of 1:2 (doubling dilutions) with PBS, to make the 12-point standard curve.

3. Coat wells A1 and B1 with 200 μ L of the first point of the IgG₄ standard curve, followed by 200 μ L of the next point in wells A2 and B2 and so on, finishing with the final point of the curve in wells A12 and B12.
4. Seal plate with parafilm and incubate for 1 h at 37 °C.
5. Wash plates 3 $\times$ with wash buffer by hand using a squeeze bottle. Once third wash is completed, tap plate onto paper towels to remove remaining buffer from the wells and dispel any bubbles.
6. Add 250 μ L of blocking buffer to each well.
7. Seal plate with parafilm and incubate for 1 h at 4 °C.
8. Wash plate 3 $\times$ with wash buffer.
9. Add 250 μ L of blocking buffer to each well.
10. Seal plate with parafilm and incubate overnight at 4 °C.
11. Wash plate 4 $\times$ with wash buffer.
12. Add 200 μ L of HRP mouse anti-human IgG₄ (diluted 1:1000 in reagent/blocking buffer) to all wells.
13. Seal plate with parafilm and incubate for 1 h at 4 °C.
14. Wash plate 6 $\times$ with wash buffer.
15. Add 100 μ L of freshly made substrate solution to each well and leave for between 5 and 10 min, until highest point of standard curve is readily visible.
16. Add 100 μ L of stop solution to each well.
17. Ensure that no air bubbles are within the wells and read plate at 450 nm absorbance.
18. Calculate average and % CV of duplicates as well as the signal-noise ratio of the positive and negative controls.
19. Plot standard curve and determine arbitrary units (AU) of mouse urine-specific IgG₄ in test samples from the standard curve based on optical density (OD) (Fig. 1).

3.2 ELISA protocol for IgG₄- Specific Antibodies to Mouse Urine

1. Coat each well (except the top two rows A1–12, B1–12) of the ELISA plate in 200 μ L of 200 μ g/mL of mouse urine extract in PBS.
2. Seal plate with parafilm and incubate for 1 h at 37 °C.
3. Wash plates 3 $\times$ with wash buffer by hand using a squeeze bottle. Once third wash is completed, tap plate onto paper towels to remove remaining buffer from the wells and dispel any bubbles.
4. Add 250 μ L of blocking buffer to each well.
5. Seal plate with parafilm and incubate for 1 h at 4 °C.
6. Wash plate 3 $\times$ with wash buffer.

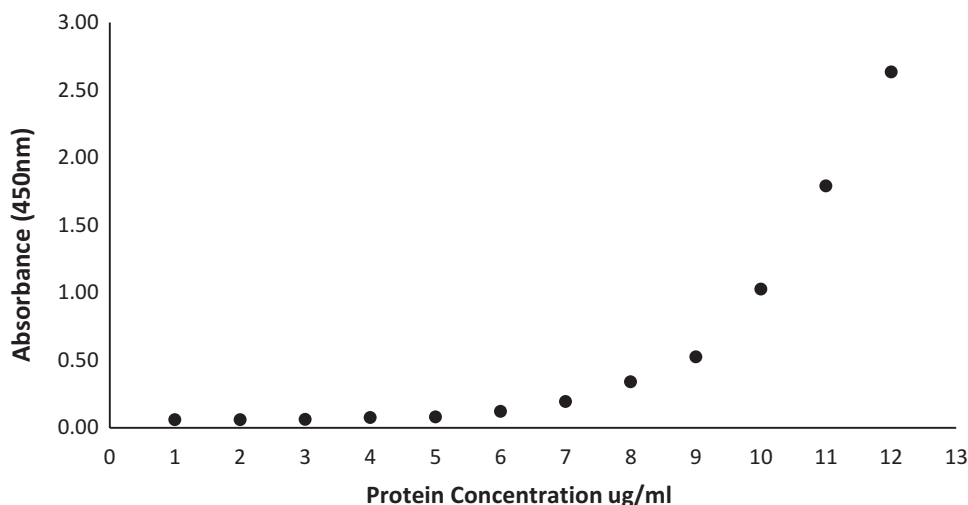


Fig. 1 Example of standard for IgG₄ ELISA. Twelve concentrations of human IgG₄ were assayed, absorbance plotted against protein concentration

7. Add 200 μ L of reagent/blocking buffer to wells C1–2. Vortex serum samples and add 200 μ L of negative control serum to wells G11–12 and positive control serum to wells H11–12. Add test serum samples, in duplicate, to all other wells of the ELISA plate.
8. Continue with ELISA as for above protocol from **step 10** onwards.

3.3 Analysis of ELISA Data

We could not obtain the required amount of analyte (mouse urine-specific IgG₄) for a standard curve. Instead, we developed a curve using human IgG₄. From the standard curve, we could determine quantities of mouse urine-specific IgG₄ and represent the values in arbitrary units (AU).

In addition, we ran a pool of positive serum and negative serum in each assay. The assays would only be accepted if the signal-to-noise ratio was above 20.

The coefficient of variation (CV) was calculated for all duplicates and those with a %CV over 20 were repeated (*see Note 2*).

4 Notes

1. Optimization of assay:
 - (a) Standard curve: Initial highest point of the curve was 1,000,000 pg/mL. When plotted, the peak of the curve was seen to be at the fourth point (125,000 pg/mL) on the curve; after this point the optical densities plateaued. Thus, for the rest of the experiments the first/highest point on the curve would be 125,000 pg/mL.

- (b) Two blocking agents were tested BSA and Chonblock, BSA performed the best of the two.
 - (c) Checkerboard analysis was performed on the serum and HRP-labeled antihuman IgG₄ to optimize the assay and stop the buildup of precipitate in the wells containing test serum samples with the highest ODs.
 - (d) To insure less variability between assays, we used a TMB substrate kit. We tested this kit with biotinylated anti-human IgG₄ and ExtrAvidin peroxidase versus HRP-labeled anti-human IgG₄. The HRP-labeled anti-human IgG₄ performed better and was therefore used throughout the assay.
 - (e) It was initially observed that even after optimization there was visible precipitate at the highest points of the standard curve. To prevent this, different blocking times were used. It was found that blocking the curve overnight prevented the formation of precipitate after substrate development.
2. Reducing % coefficient of variance (CV) between duplicates:
- (a) After addition of reagents to wells tap plate for 1 min (before incubation) to ensure that all reagents are mixed well.
 - (b) Pre-wet pipette tips 2–3 times in required solution before pipetting.
 - (c) Make up new reagent/blocking buffer and wash buffer before every experiment. Make up coating, standard curve, serum, and HRP-labeled anti-human IgG₄ dilutions immediately before use.
 - (d) Avoid exposing the plate to light during the incubation of the HRP-labeled anti-human IgG₄.
 - (e) The optical density of the highest point in the curve should be between 2 and 3 OD_{450nm} once developed.
 - (f) During incubation, ensure that plates are placed on a flat surface and temperature stays constant throughout the experiment (i.e., use fridge and incubator that maintain constant temperatures).
 - (g) Ensure after each wash step that there are no bubbles left in the wells.

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