



Isotype-Specific Detection of Serum Immunoglobulins Against Allergens

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Abstract

Type-I hypersensitivity is commonly characterized by increased levels of antigen-specific immunoglobulin (Ig) E. Therefore, it is important for clinical and research investigators to reliably measure serum levels of IgE in allergic patients and animal models. While current ELISA-based methods are simple and commonly performed for the detection of allergen-specific IgE using serum or plasma, they may produce misleading results. This is in part due to decreased sensitivity for IgE in the presence of other Ig isotypes in the same sample, such as IgG, that are typically more abundant than IgE. When assessment of multiple Ig isotypes is necessary, performing optimized assays for individual isotypes requires high sample volumes. Here, we describe an approach to increase the sensitivity for IgE detection while conserving the sample volume needed. This method not only improves the accuracy of serum IgE measurements but also allows simultaneous analysis of other allergen-specific immunoglobulins.

Key words Adsorption, Allergy, Enzyme-linked immunosorbent assay (ELISA), Immunoglobulins, Protein-G

1 Introduction

Allergic sensitization results from inappropriate recognition and subsequent presentation of normally benign substances as harmful antigens. This process is mediated by the major histocompatibility complex II (MHC-II), a receptor complex expressed by professional antigen presenting cells such as dendritic cells and macrophages. MHC-II forms a complex with a processed antigen peptide and presents them to CD4+ T cells, which in turn induce isotype class switching in B cells for antigen-specific immunoglobulin (Ig) E production [1]. The produced IgE bind to the Fcε receptors on mast cells and basophils for rapid release of histamine, cytokines, and other inflammatory factors upon re-exposure to the allergen, causing swelling and redness commonly associated with type-I immediate hypersensitivity reactions [2]. Because serum IgE concentrations are normally very low, testing allergic individuals for

elevated levels of IgE for particular antigens is useful in identifying offending allergens. This test is commonly practiced in clinics for diagnosis of allergy in addition to skin prick tests [1].

However, detection of other Ig isotypes, especially IgG and IgA, can also provide important information for understanding the development and progression of allergy. While IgG is classically associated with delayed type hypersensitivity reactions, IgG1, IgG2a, and IgG2b subclasses have been shown to potentially contribute to or independently cause anaphylaxis in experimental studies and possibly allergic individuals [3]. In contrast, an increase of serum antigen-specific IgG4 has been implicated in allergy resolution [4]. Additionally, individuals with decreased IgA are thought to be more susceptible to development to various allergies [5]. While the precise roles of these other Igs in allergy development are still elusive, IgE levels alone may not provide a complete measure of humoral responses in allergic individuals.

The enzyme-linked immunosorbent assay (ELISA) has become a widely used method to detect a variety of Ig isotypes over the past decades. However, the assay may produce biased and unreliable outcomes if the presence of allergen-specific IgG is predominant and competes for the same antigen against a relatively small amount of allergen-specific IgE in the same serum sample [6] (Fig. 1). Additionally, when multiple Ig isotype levels are to be measured in smaller animals such as mice, it is often not feasible to dedicate a large volume of serum to carry out the assays for multiple Ig isotypes individually. Therefore, the current ELISA approach may not be optimal in some cases, especially when the Ig isotype of interest is present in low abundance.

Here, we outline a method to separate Ig isotypes from a single serum sample by adsorbing total IgG using magnetic protein G beads to provide a more accurate measure of IgE in the sample by ELISA (Fig. 2). Since each serum sample is highly diluted prior to the separation process, only 6 μ L of serum is required to individually assay multiple Igs. Moreover, the adsorbed IgG can be subsequently eluted and used for detection of total or allergen-specific IgG using a standard ELISA protocol with minor modifications.

2 Materials

Use ultrapure water (18.2 Ω) and analytical grade reagents. Prepare and store all reagents at 4 °C unless indicated otherwise. Do not add sodium azide to buffers.

2.1 Preparation of Serum Samples

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4. Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ in 800 mL water. Adjust pH to 7.4 and add water to a final volume of 1 L. Store at room temperature.

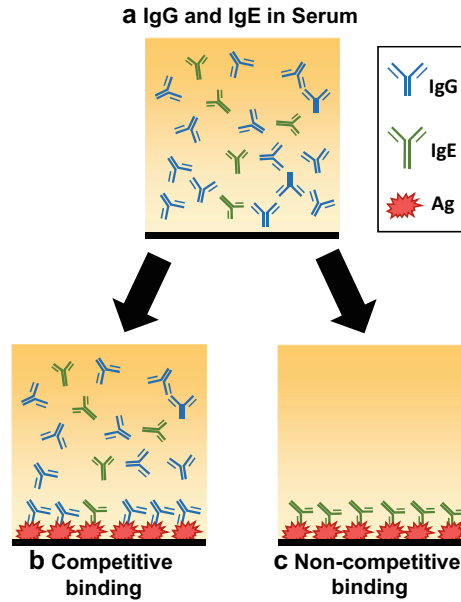


Fig. 1 Potential masking of IgE by IgG during ELISA. IgE is present in a relatively low amount in serum samples compared to IgG (a). When untreated serum is used for ELISA, antigen-specific IgG competes for the antigen binding sites (b). Pretreatment of serum samples with protein G adsorption removes the IgG, allowing antigen-specific IgE to bind to the antigen (c)

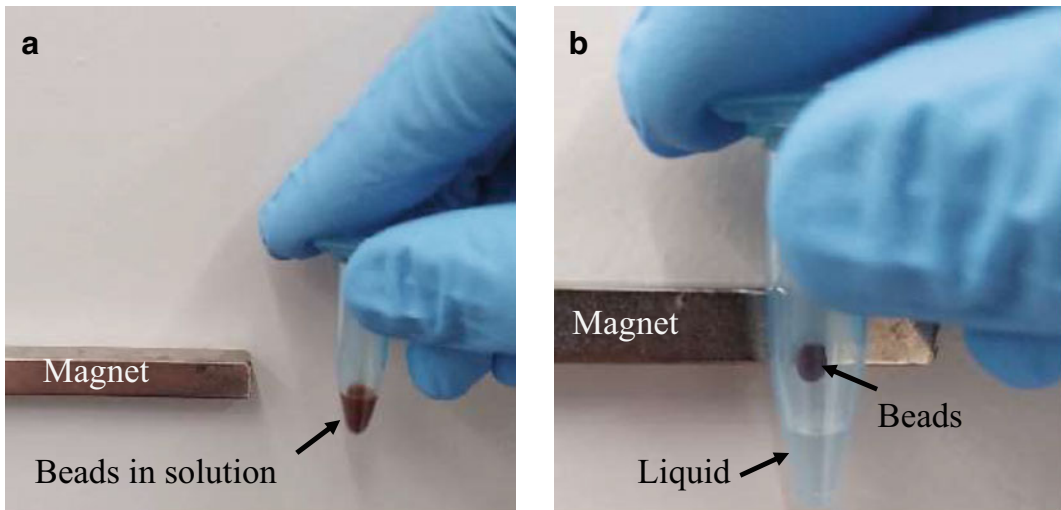


Fig. 2 Separation of protein G beads with a magnet. After vortexing, protein G beads are well suspended in the solution (a). With a magnet applied to the side of the tube, the beads become easily separated from the solution (b). Remove the solution with a pipette and prepare the beads as described in Subheading 3.1.1

2. Assay buffer: 0.05% (v/v) Tween-20 and 0.5% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4. Add 500 μ L of Tween-20 and 5 g of BSA in 1 L of PBS.
3. 0.5-mL Microfuge tubes.
4. Magnetic protein G beads (*see* **Note 1**).
5. Magnet.
6. Vortexer.
7. Elution buffer: 50 mM Glycine-HCl, pH 2.8. Dissolve 0.38 g of glycine in 80 mL of H₂O. Adjust pH and add water to a final volume of 100 mL.
8. Tris buffer: 1 M Tris-HCl, pH 7.5. Dissolve 12.1 g Tris in 80 mL of H₂O. Adjust pH and add water to a final volume 100 mL.

2.2 Antigen-Specific IgE/IgG ELISA

1. High-binding 96-well ELISA plate or strips: clear, flat-bottom.
2. Coating buffer: 44.0 mM NaHCO₃, 6.04 mM Na₂CO₃, pH 9.6. Add 3.7 g NaHCO₃ and 0.64 g Na₂CO₃ to 1 L of water.
3. 100 $\times$ Target antigen stock solution: 2 mg/mL in the coating buffer for coating ELISA plate (*see* **Note 2**). Dissolve 10 mg target antigen in 5 mL the coating buffer (*see* **Note 3**).
4. 10-mL Syringe.
5. 0.2- μ m Syringe filter.
6. PBS: *See* Subheading 2.1, **item 1**.
7. Wash buffer: 0.05% Tween-20. Add 500 μ L of Tween-20 to 1 L of PBS.
8. Assay buffer: *See* Subheading 2.1, **item 2**.
9. Species-specific biotinylated secondary antibody.
10. Horseradish peroxidase-conjugated avidin (HRP-avidin).
11. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution: Commercially available stabilized TMB solution.
12. Stop solution: 2 N H₂SO₄.
13. Micropipettors: multichannel pipettors optional.
14. Pipette tips.
15. Plate sealers.
16. Plate rocker or orbital shaker.
17. Microplate reader capable of reading at 450 nm.

3 Methods

3.1 Preparation of Serum Samples

This section describes a method to separate IgG from serum samples to be used for antigen-specific IgE detection by ELISA. To avoid freezing and thawing of the samples, this brief procedure should be performed when an ELISA plate has been coated and ready to be used, or when a pre-coated ELISA plate is used.

3.1.1 Adsorption of IgG from Diluted Samples

1. If frozen aliquots of serum samples are being used, thaw quickly and keep on ice until diluted in the assay buffer.
2. Dilute 6 μL of each serum sample with 234 μL of the assay buffer to a total volume of 240 μL and set aside (*see Note 4*).
3. Vortex magnetic protein G beads in the original container for 1 min and then immediately transfer 50 μL of the beads into a 0.5-mL microfuge tubes. Place a magnet on the side of the tube and wait briefly for beads to aggregate on the tube wall (Fig. 2). Discard the liquid, leaving only the beads in the tube.
4. Add the diluted serum from **step 2** to the beads from **step 3** and close the lid tightly. Vortex briefly for 3 s and place the tube on a rocker to keep beads agitated in suspension for 10 min at room temperature.
5. Place the magnet on the side of the tube and collect the supernatant (Fig. 3a), which is now devoid of IgG (Fig. 3b). Transfer the supernatant in a clean tube and save for allergen-specific IgE ELISA described in Subheading 3.2 (*see Note 5*). If performing IgG detection, immediately proceed to Subheading 3.1.2 to elute IgG adsorbed by the beads (*see Note 6*).

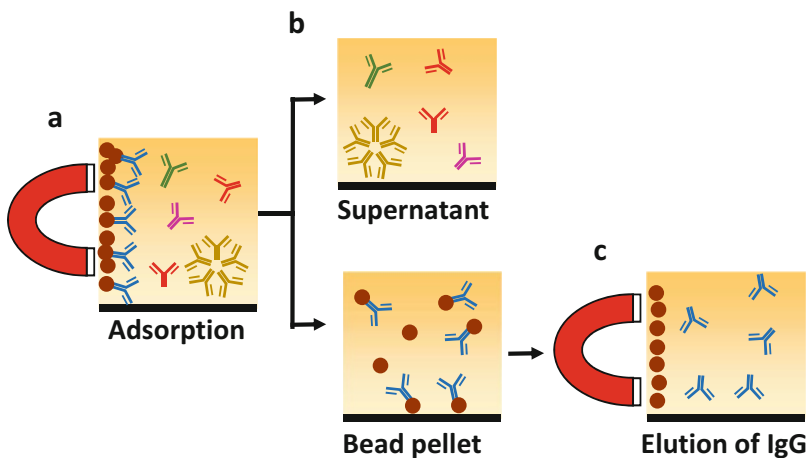


Fig. 3 Adsorption of total immunoglobulin G. A diluted serum sample is added to magnetic protein-G beads. The beads bind to IgG and are subsequently pelleted with a magnet (a). The remaining supernatant, which contains IgE but not IgG (b), is collected and used for total or antigen-specific IgE ELISA (b). Bead-bound IgG is immediately eluted with a low-pH buffer, neutralized, and used for total or antigen-specific IgG ELISA (c)

3.1.2 Elution of IgG from Magnetic Beads

1. Immediately add 200 μL of wash buffer to the beads from Subheading 3.1.1, **step 5**. Gently mix by pipetting to avoid foaming.
2. Place the magnet to the side of the tube and discard the wash buffer. Immediately add 25 μL of elution buffer to the beads and gently mix by pipetting. Incubate for 2 min at room temperature.
3. Neutralize the elution buffer with 25 μL of Tris buffer and mix well (*see Note 7*). Adjust the volume to 240 μL with the assay buffer and collect the IgG-containing supernatant in a clean microfuge tube (Fig. 3c). Keep on ice until proceeding to antigen-specific IgG ELISA described in Subheading 3.2.

3.2 Detection of Antigen-Specific IgE or IgG with ELISA

3.2.1 Preparation of ELISA Plate with an Antigen

1. If a nonsterile 96-well ELISA plate or 8-well ELISA strips are used, sterilize under ultraviolet light in a biological safety cabinet (*see Note 8*).
2. Dilute the 100 $\times$ antigen stock solution with coating buffer to make 1 $\times$ antigen-coating solution. For enough 1 $\times$ antigen-coating solution for one 96-well plate, dilute 100 μL of the 100 $\times$ antigen stock solution in 10 mL of the coating buffer. Sterile filter the 1 $\times$ antigen-coating solution using a 0.2- μm filter attached to a syringe.
3. Pipette 100 μL of the 1 $\times$ antigen-coating solution to all wells. Seal the plate and incubate on a rocker with gentle agitation overnight at 4 $^{\circ}\text{C}$.
4. Remove the antigen-coating solution and rinse wells with 300 μL of the wash buffer three times, 1 min each. Blot dry between the rinses by tapping the inverted plate on an absorbent surface, such as a stack of paper towels (*see Note 9*).
5. Place 200 μL assay buffer in each well to block nonspecific binding. Seal the plate and incubate on a rocker for 2 h at room temperature.
6. Remove the assay buffer from the wells and place 100 μL of prepared samples from Subheading 3.1.1 (for IgE) or Subheading 3.1.2 (for IgG) in their designated wells (*see Note 10*). Seal plate and incubate overnight at 4 $^{\circ}\text{C}$.

3.2.2 Colorimetric Reaction and Plate Reading

1. Aspirate serum samples and wash wells with 300 μL of wash buffer three times, 1 min each. Blot dry between the rinses as described in Subheading 3.2.1, **step 4**. Place 100 μL of a biotinylated secondary antibody at a working concentration in each well (*see Note 11*). Seal the plate and incubate on a rocker with gentle agitation for 2 h at room temperature.

2. Remove the secondary antibody solution and rinse wells with wash buffer four times, 1 min each. Blot dry between the rinses. Add 100 μL of HRP-avidin at a working concentration to each well (*see Note 11*). Seal the plate, protect from light, and incubate on a rocker with gentle agitation for 1 h at room temperature.
3. Remove HRP-avidin and rinse the wells with the wash buffer three times, 1 min each.
4. Place 100 μL of TMB substrate solution (*see Note 11*) to each well and incubate on a rocker for 20–30 min, protected from light (*see Note 12*).
5. Stop the color reaction with 100 μL of the stop solution and immediately read the absorbance at 450 nm on a microplate reader. If possible, additionally read with a reference wavelength at 540–570 nm (*see Note 13*). Example of results are shown in Fig. 4.

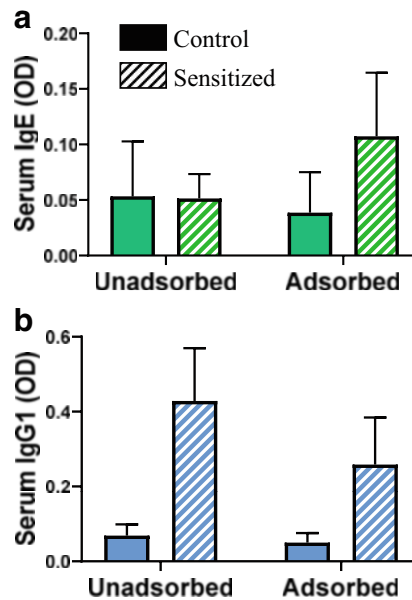


Fig. 4 Comparison of allergen-specific IgE and IgG1 ELISAs using adsorbed and unadsorbed serum samples. Male C57BL6/J mice were orally sensitized to a cow's milk protein, β -lactoglobulin. Allergen-specific IgE and IgG1 ELISAs comparing the sera from sham control and sensitized mice were performed without or with the IgG adsorption pretreatment. The difference in the IgE levels between the control and sensitized mice was more evident with the pretreatment (**a**) while the relative differences observed for IgG1 with the eluted sera remained unchanged (**b**). Mean $\pm$ SEM, $n = 5$. OD optical density

4 Notes

1. This protocol describes the use of magnetic protein G beads, which bind to all IgG isotypes with high affinity. However, other protein bead options, such as protein A beads, that show variable affinities to other antibody isotypes are also available [7].
2. An important factor in coating the plate adequately with a given antigen is the pH of the buffer used. The pH of the buffer facilitates the attachment of the antigen to the microplate and thus has a significant effect on the strength of the color reaction. The most common buffer used is a bicarbonate buffer (pH 9.6), although PBS (pH 7.4) is occasionally used. If the color reaction is weak, adjusting the pH of the coating buffer may facilitate better binding of some antigens. Optimal coating conditions should ultimately be determined for specific antigens used by individual laboratories.
3. The antigen concentration of 2 $\mu\text{g}/\text{mL}$ (using the $1\times$ stock solution) has been optimized for the detection of IgE against a purified bovine milk whey protein, β -lactoglobulin (Bos d 5) [8]. Optimal coating concentrations of other antigens may vary and therefore need to be determined by individual laboratories. For example, we have used 20 $\mu\text{g}/\text{mL}$ to coat plates with bovine whey proteins [9], while others have used up to 50 $\mu\text{g}/\text{mL}$ of antigens to coat plates with shrimp or peanut extract [6].
4. While 6 μL of serum is a sufficient volume for the detection of IgE against β -lactoglobulin in our orally sensitized mouse model of milk allergy, a greater amount of sera may be necessary depending on the mouse model being investigated. The optimal serum concentration for ELISA should ultimately be determined by each laboratory.
5. These aliquots may be kept on ice until the IgG elution procedure is completed. Avoid repetitive freezing and thawing of the samples.
6. If adsorbed IgG needs to be eluted from the beads, immediately proceed to Subheading 3.1.2 and add wash buffer to the remaining beads. Do not leave the beads to dry.
7. With a short incubation time, a repeating pipettor is a convenient tool to add neutralization buffer quickly.
8. We perform ELISA plate coating under a sterile condition to avoid potential microbial growth during the coating and sample incubation processes. Microbial growth can alter Ig binding and the later development of the color reaction.

9. Care should be taken to remove as much of the wash buffer as possible without completely drying out the wells. Firmly tapping the plate 3–4 times on a stack of paper towels laid on a laboratory bench should be sufficient.
10. The supernatants from the protein G adsorption should yield enough (approximately 200 μ L) to run each sample in duplicates. While the postadsorption supernatant can also be used to detect serum IgA, it should not be used for IgM ELISA since this isotype is partially absorbed by protein G.
11. Use working concentrations of secondary antibodies and HRP-avidin as suggested by the suppliers. However, optimal concentrations for these reagents to detect each Ig should ultimately be determined by individual laboratories.
12. For an initial reaction, periodically inspect the development of the yellow reaction product to avoid color saturation.
13. The reference wavelength can help to correct for optical imperfections in the wells, and the reading can be subtracted from the measurements at 450 nm.

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