

The Facilitated Antigen Binding (FAB) Assay – A Protocol to Measure Allergen-Specific Inhibitory Antibody Activity

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Abstract

Specific allergen immunotherapy is an effective treatment for IgE-mediated allergic disease and involves T- and B-cell mediated events. IgE receptors on the surface of antigen-presenting cells facilitate the presentation of allergens in the presence of specific IgE antibody resulting in T-cell activation. Interference with these IgE-dependent mechanisms by 'blocking' IgG antibodies may downregulate T-cell responses and manifest as a reduction in allergic responses in vivo.

The vigor of proliferative responses by T-cell clones is representative of the binding of allergen–IgE complexes to B cells. Therefore, a simplified assay can be employed that measures the binding of allergen–IgE complexes to B cells instead of a more complex assay involving proliferative assays using antigen-specific T-cell clones. Allergen–IgE complexes can be easily detected by flow cytometry and this simplified technique is called the IgE-facilitated allergen binding (IgE-FAB) assay which is described in this chapter.

Key Words: T cell; B cell; immunotherapy; flow cytometry; IgG.

1. Introduction

Specific allergen immunotherapy is an effective treatment for IgE-mediated allergic disease (1). The mechanisms that are associated with successful immunotherapy involve both T- and B-cell mediated events (2). Cellular changes include immune deviation favoring T helper 1 responses (3) and the induction of regulatory cells producing interleukin (IL)-10 (4). Treatment is also associated with the induction of allergen-specific serum IgG4 antibodies (5–7). IgG4 antibodies are thought to have blocking activities as they compete with IgE for allergen binding to mast cells, basophils, and other IgE receptor-expressing cells (8).

Receptors for IgE expressed on the surface of antigen-presenting cells have been shown to facilitate the presentation of allergens in the presence of specific

IgE antibody resulting in effective T-cell activation at low concentrations of allergen (9). Interference with these IgE-dependent mechanisms by 'blocking' IgG antibodies may downregulate T-cell responses and manifest as a reduction in allergic responses in vivo.

Previous studies by van Neerven and colleagues (10,11) have shown that serum obtained from subjects receiving birch immunotherapy was able to inhibit IgE-facilitated presentation of allergen by B cells to an allergen-specific T-cell clone. Another study has also demonstrated that serum obtained from a double-blind placebo-controlled trial of grass pollen immunotherapy can inhibit IgE-facilitated allergen presentation to a grass-specific T-lymphocyte clone (12). This report demonstrates that the vigor of proliferative responses by T-cell clones is representative of the binding of allergen-IgE complexes to B cells. Therefore, a simplified assay can be employed that measures the binding of allergen-IgE complexes to B cells instead of a more complex assay involving proliferative assays using antigen-specific T-cell clones. Allergen-IgE complexes can be easily detected by flow cytometry and this simplified technique is called the IgE-facilitated allergen binding (IgE-FAB) assay.

Briefly, the IgE-FAB assay involves incubation of serum, known to contain high levels of allergen-specific IgE, with low concentrations of allergen extract in the presence or absence of 'test serum' with potential inhibitory activity. The resulting allergen-IgE complexes are subsequently incubated with an EBV-transformed human B-cell line. These cells express high levels of the low-affinity IgE receptor (CD23, FcεRII) which binds allergen-IgE complexes. Detection of allergen-IgE complexes on the surface of B cell can be achieved using either a biotinylated allergen or a fluorescent antibody against IgE and analyzed using flow cytometry (Fig. 1). In the presence of inhibitory serum, number of allergen-IgE complexes bound to B cells is decreased.

2. Materials

2.1. Assay Buffers

2.1.1. FAP Buffer (pH 7.2)

8.10 g NaCl, 0.16 g NaH₂PO₄, 0.98 g Na₂HPO₄, 1.0 g BSA (0.1%) (all from Sigma or any qualified commercial company). Dissolve in 1 L of distilled H₂O, adjust pH to 7.2. Store for up to 2 wk at 4°C.

2.1.2. EBV Growth Medium

RPMI 1640 supplemented with 1% (v/v) L-glutamine (Invitrogen, Paisley, UK), 10% heat-inactivated FCS (PAA laboratories, Yeovil, UK), and 1% (v/v) penicillin/streptomycin mixture (Invitrogen). Store at 4°C for up to 4 wk.

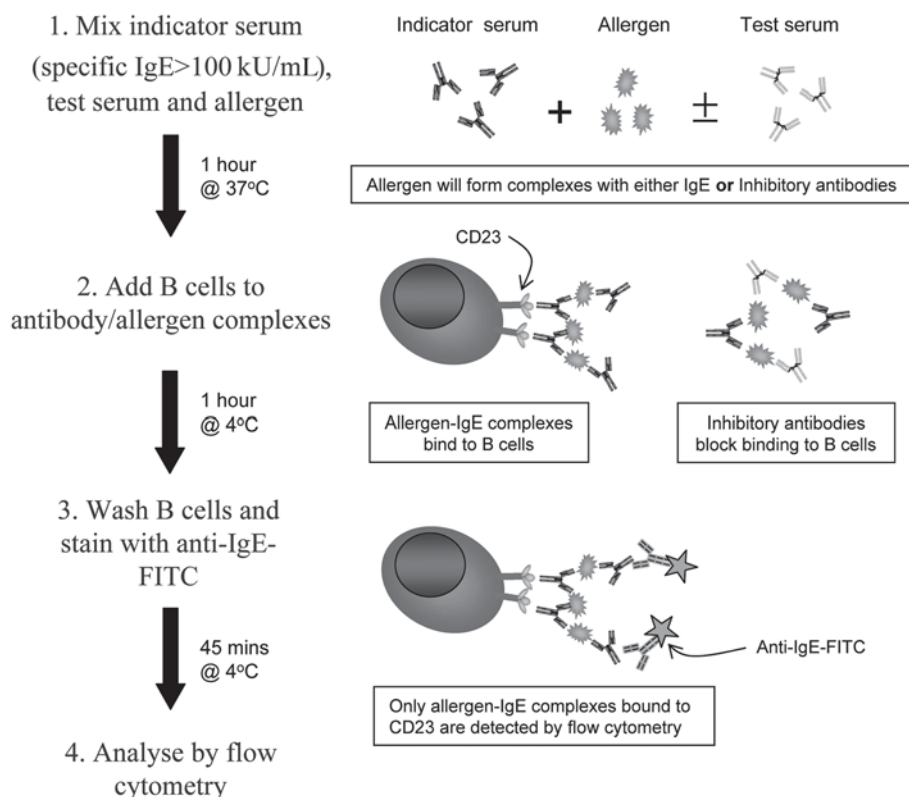


Fig. 1.

2.2. Serum and Allergen Requirements

- Indicator serum should be obtained from an atopic (allergic) donor with high allergen-specific IgE levels (as measured by CAP-RAST). Serum with levels of >100 kU/L are generally found to be suitable indicator serum as the level of binding to EBV-transformed B cell is proportional to the amount of IgE contained within the serum. If serum with >100 kU/mL is not available then it is worthwhile testing serum with 50–100 kU/mL. Generally, serum with less than 50 kU/mL does not bind in the assay. Serum (plasma) from allergic donors can also be purchased from Plasmalabs, Washington.
- Inhibitory serum to be tested in the assay can be obtained from subjects undergoing a specific treatment or with signs of clinical remission/tolerance. Serum can be collected using standard blood tubes with clotting agent.
- Allergen can be purchased from a number of commercial companies or isolated by standard laboratory methods. Freeze-dried allergen should be dissolved in sterile RPMI at 1–5 mg/mL, aliquotted, and stored at –20°C.

3. Methods

3.1. Cultivation of EBV-Transformed B Cells

Detailed description of the generation of EBV-transformed B cells is beyond the remit of this protocol. Briefly, supernatant from a EBV-producing cell line (e.g., B-958) is cultured with peripheral blood mononuclear cells (PBMC) from an atopic donor (allergic to the allergen of interest). Transformed B cells are enriched for high CD23 expression by the use of magnetic bead separation and stored in liquid nitrogen.

1. EBV-transformed B cells are rapidly thawed at 37°C and washed twice in 50 mL of RPMI.
2. The cell line is maintained in vented-cap, canted-neck cell culture flasks from Falcon/VWR (Poole, UK) at 37°C, 5% CO₂, and 95% relative humidity (RH).
3. Cells should be subcultured two to three times a week to maintain cell density of 0.5–1.5 × 10⁶ cells/mL.
4. Cell viability should be maintained above 95%.
5. Cells can be used in the assay a minimum of 10–14 d after the start of the culture period and up to a maximal of 60 d.
6. CD23 expression on B-cell lines should be routinely checked by flow cytometry, e.g., using CD23-FITC (DakoCytomation). Numbers of B cells expressing CD23 should be very high (>98%) and levels of expression should lie within 10² and 10³ MCF.

3.2. Analytical Procedure

3.2.1. Serum Preparation

Serum stored at –80°C should be left for 24 h at –20°C, then thawed in a water bath at 37°C for 10 min. Serum containing precipitants may be filtered before use in the assay.

3.2.2. Allergen Dilutions

Allergen should be freshly prepared in RPMI to a working dilution 10 × greater than the required final concentration, e.g., if a final allergen concentration of 1 µg/mL is required the substock solution should be prepared at 10 µg/mL.

3.2.3. Complexing Allergen with Serum Antibodies

1. 20 µL of serum, the inhibitory activity of which is to be tested, is added to a tube (BD Falcon 5-mL polystyrene round-bottom tube). Control tubes should contain 20 µL of RPMI.
2. Add 20 µL of ‘high-IgE’ indicator serum.
3. Add 5 µL of the allergen dilution.
4. Place a cap on the tube to minimize evaporation.
5. Incubate serum/allergen for 1 h at 37°C.

3.2.4. Preparation of EBV-B Cells

1. Transfer cells from culture flask into a 50-mL centrifuge tube.
2. Wash cells by centrifugation at 490g at 4°C for 7 min.
3. Repeat wash step with 50 mL cold RPMI (centrifuge at 490g at 4°C for 7 min).
4. Resuspend cells in 10 mL of RPMI.
5. Count cells by trypan blue exclusion (1:1 dilution: 20 μ L cell suspension in 20 μ L trypan blue) using a hemocytometer. Duplicate sampling for cell counts is recommended.
6. Centrifuge the cells and resuspend to 2×10^7 cells/mL in FAP buffer.
7. Store cells on ice and mix thoroughly prior to use.

3.2.5. Incubation of EBV-B Cells with Precomplexed Allergen

Add 5 μ L of EBV-B cell solution (100,000 cells per test) to allergen–serum complexes. Mix gently and incubate for 1 h on ice.

3.2.6. Staining of Allergen–IgE Complexes Bound to B Cells (see **Note 1**)

1. Wash B cells from the previous step twice with 1 mL of FAP buffer (centrifuge at 490g at 4°C for 7 min).
2. Dilute anti-IgE-FITC antibody 1:50 (e.g., 20 μ L in 980 μ L of FAP buffer).
3. Add 20 μ L of diluted anti-IgE-FITC antibody to each tube.
4. Incubate for 45 min on ice, in the dark.
5. Wash cells 1 mL per tube and resuspend in 200 μ L of ice-cold FAP buffer.
6. Analyze surface staining immediately on flow cytometer.

3.2.7. Acquisition Using the Flow Cytometer

1. Open forward scatter vs side scatter dot-plot and identify EBV cell population as the main population.
2. Gate around the population in FSC/SSC and adjust the FL-1 gain so that cells appear between 10^0 and 10^1 on a histogram plot.
3. Ensure that a positive signal is detected using the indicator serum-positive control (see below).
4. Acquire 5000 gated cells per sample.
5. The following controls should be included in each experiment:
 - a. Cells only (i.e., no FITC stain; however, cells should be incubated with human serum as this can increase fluorescence).
 - b. Cells + isotype control for IgE-FITC.
 - c. Cells + IgE-FITC.
 - d. Indicator serum/allergen + IgE-FITC (serum-positive control).
 - e. Internal negative control (for inhibition).
 - f. Internal positive control (for inhibition).

3.2.8. Running Multiple Samples

The above protocol uses standard 5-mL Falcon ‘FACS’ tubes in the assay. For multiple samples, a 96-well U-bottomed plate (Nunc) may be used. In this

case, a centrifuge equipped with microplate carriers is required and wash steps should be carried out using a multichannel pipet. In addition, wash volumes should be reduced to 250 μL (or so that the maximal volume of the well is not exceeded). For FACS analysis, contents of the wells should be mixed well and transferred to microtubes (Bioquote, UK). Microtubes can be placed in a standard FACS tube and used as normal.

3.2.9. Optimization of the Assay

It is essential to optimize the FAP assay for your allergen of choice. The following experiments should be completed before using the assay on large numbers of test samples:

1. Identification of a suitable indicator serum (containing high allergen-specific IgE levels, *see* **Section 2.2.**). Potential serum to be used as indicator serum should be first tested for binding with a limited dose–response of allergen (e.g., 0.01, 1, and 100 $\mu\text{g/mL}$) without inclusion of inhibitory serum.
2. Any serum showing binding of >30% (at any allergen concentration) should be further tested using a more comprehensive dose–response curve (e.g., half logarithmic doses from 1 pg/mL to 100 $\mu\text{g/mL}$). Typical optimal binding occurs at 0.1–5 $\mu\text{g/mL}$. It is desirable to achieve binding of >50% although binding in the range of 70–80% is optimal. Indicator serum binding of approx. 30% can be used in the assay although increased variability is observed.
3. Once a suitable indicator serum is identified, the time course of allergen–IgE complex binding to B cells should be optimized (*see* **Section 3.2.5.**). Binding time between 2 and 120 min can be tested (e.g., 2, 10, 30, 60, and 120 min).
4. If indicator serum is of limited availability, dilutions (in RPMI) of serum can be tested for binding in the assay. Also, pooled serum can be used in place of serum from a single donor.
5. If serum with potential inhibitory activity is available, this should be tested in the assay before running large numbers of test samples. The assay should be run using a dose range of allergen concentrations in the presence or absence of potential inhibitory serum. As inhibition of FAP occurs at low allergen concentrations, an allergen concentration should be chosen that generates binding of >50% (or less, *see* above) but also allows the maximal inhibition of binding by test sera.

4. Notes

1. Allergen–IgE complexes can also be detected using biotinylated allergen. In such a case optimal binding/inhibition should first be established using the biotinylated allergen. Detection of complexes (4.6) is achieved using streptavidin-PE (BD biosciences).

Acknowledgment

The author would like to thank Drs Peter Wurtzen, Petra Wachholz, and Mohamed Shamji for their contributions to this protocol.

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