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Technical Note: A simple method for measuring human cell-bound IgE levels in whole blood

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Abstract

IgE plays a critical role in hypersensitivity reactions such as asthma and allergy as well as poorly defined roles in immunity to parasitic helminth infections. The quantity of antigen-specific IgE is thought to affect the intensity of the allergic reaction as well as the perceived level of resistance to parasitic worms. Because most somatic IgE is bound by its receptors, FcεRI and FcεRII, and increased expression of IgE receptors also change with cellular activation status, the serum concentration of IgE may not necessarily reflect levels of systemic IgE. Accurate measures of IgE would help to define the bona fide role of this molecule in immunity. Furthermore, improved indicators of systemic antigen-specific IgE could better predict the risk for severe allergic responses. In this report, we demonstrate a simple method for measuring cell-bound IgE in whole blood using basic flow cytometry. This method demonstrates that, in general, cell-bound IgE correlates well with serum levels of IgE. However, discordance in serum and cell-bound IgE levels in some individuals illustrates the inadequacy of using serum levels of IgE as a systemic indicator and validates the need to assay both cell-bound and free IgE.

Keywords

IgE; human; FcεRI; FcεRII; helminth; allergy

Introduction

Although IgE has the propensity to induce allergy, a seemingly evolutionary insignificant, annoying, and potential life-threatening function, IgE likely evolved to perform important immunological functions. IgE is elevated during parasitic worm infections and probably plays a host protective role, although the mechanisms remain undefined (Dunne DW, 1992). Challenges in defining the bona fide role of IgE in immunity are many and include inherent differences in human IgE receptor expression from the murine immune system. For example, human eosinophils express IgE receptors but murine eosinophils do not (de Andres et al., 1997). The *in vivo* functionality of IgE therefore remains poorly understood in humans. The quantity of antigen-specific IgE is thought to affect the intensity of an allergic reaction as well as the perceived level of resistance to parasitic worms (Dunne DW, 1992; Sampson and Ho, 1997). IgE exerts its effector functions through cell expressed IgE receptors, FcεRI and

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FcεRII. Thus, levels of free IgE molecules do not necessarily reflect the concentrations of somatic IgE as the majority of IgE is bound by high affinity IgE receptors (FcεRI) on tissue mast cells. Expression of IgE receptors may be altered by the cellular activation status of the cell which is influenced by multiple immunological factors (Mawhorter et al., 1996; Ganley-Leal et al., 2006). IgE engagement by its cellular receptors could confound measurements of IgE, thereby blurring the interpretation of the role of IgE in immunity.

An accurate measure of IgE, both free and cell-bound, is essential for understanding the elusive role this molecule might have in mediating immunity to parasites as well as defining at-risk individuals for severe allergic reactions. Current methods to measure IgE include assaying serum IgE levels by standard ELISA or by assessing the intensity of the allergic reaction via skin prick test or RASTS (Glovsky, 2007). While skin pricks and IgE measurements are a relatively specific indication of environmental allergies, they are not reliable for food allergies (Rance et al., 2002). The double-blind, placebo-controlled food challenge (DBPCFC) is the current “gold standard” for the diagnosis of food allergy. However, this test is costly and puts the patient in potential danger of anaphylaxis (Rance et al., 2002). Thus, more accurate measures of systemic IgE levels could eliminate the need to perform DBPCFC. Another important application for precise IgE measurements is for patients who have been treated with anti-IgE Fc, Omalizumab® or Xolair®, which blocks binding of IgE to its receptors. Mechanisms to measure cell-bound IgE would help validate the efficacy of this drug (Hamilton et al., 2005) and any long term immunological consequences of this therapy (Hjelm et al., 2006).

Multiple cells in peripheral blood express IgE receptors and bind IgE. We have developed a simple method to measure FcεR-bound IgE in whole blood using flow cytometric techniques. In general, serum concentrations correlated well with levels of cell-bound IgE. However, in some cases, serum IgE concentrations were not reflective of cell-bound levels. Thus, using the measure of free IgE molecules in serum is an inadequate predictor of systemic IgE levels and validates the need to assay both cell-bound IgE and free IgE.

Materials and Methods

Reagents

For the flow cytometry

2 ml of sodium heparin-anticoagulated peripheral blood.

1X FACS lysis buffer (BD Pharmingen)

Cold FACS buffer: 0.1% Bovine serum albumin (BSA) in phosphate buffered solution (PBS)

Facs tubes (5 ml polystyrene tubes)

Anti-FcεRII (CD23) PE (BD Pharmingen)

Anti-IgE (BD Pharmingen)

Anti-FcεRI alpha chain (ebioscience)

Appropriate isotype control for anti-IgE (BD Pharmingen)

The assay requires at the minimum a 3 color flow cytometer. The following protocol was implemented on a 3 color FACScan (Beckon Dickinson).

Flow data analysis software. The data presented here was generated with WinMDI which can be downloaded free-of-charge at www.scripps.edu.

For the ELISAs

Immunolon ELISA plates (Fisher)

Carbonate buffer (0.5 M sodium carbonate/0.35 M sodium bicarbonate carbonate buffer, pH 9.6)

Blocking buffer (1% BSA in PBS)

Dust mite antigen (Indoor Biotechnologies)

Capture anti-IgE (Sigma)

Detection anti-IgE biotinylated (Southern Biotech)

IgE for standard curve (Diatec or Serotec)

Streptavidin (Sigma)

TMB (BD Pharmingen)

Stop solution (1 M H₂SO₄)

ELISA plate reader

Flow cytometry assay

Add 100 µl of whole blood to the bottom of 2 FACS tubes. Place the rest of the blood aside for plasma collection. Add in 2 ml of 1 × FACS lysis buffer to lyse red blood cells. Cover and incubate at room temperature for 30 minutes. When incubation is complete, centrifuge tubes at 1200 rpm for 5 minutes and pour off supernatant. Resuspend pellet in 100 µl of FACS buffer. For this protocol we use the following combination of antibodies: In tube 1, 10 µl of anti-CD23 PE, 10 µl of anti-FcεRI FITC, and 2 µl of IgE biotin. In tube 2, 10 µl of anti-CD23 PE, 10 µl of anti-FcεRI FITC, and 2 µl of biotinylated isotype control. Incubate at 4°C for 30 minutes. Wash with 2 ml of cold FACS buffer and centrifuge as above. Add 100 µl of 1:400 strep-avidin cy-chrome diluted in FACS buffer. Incubate for 10 minutes at 4°C in the dark, wash, and resuspend in 300 µl of PBS.

To identify IgE+ receptor bearing cells, anti-CD19 may be used to identify B cells, anti-CD63 for basophils, and anti-CD125 for eosinophils (all from BD Pharmingen). Spin remaining blood to collect plasma and freeze at -20°C until use.

ELISA to measure total and antigen-specific IgE in serum

For demonstration purposes, a standard ELISA format to assay levels of IgE in the plasma is utilized. Level of dust mite-specific IgE and total polyclonal IgE are measured. To measure dust mite specific IgE, ELISA plates are coated with 5 µg/ml of dust mite antigen in carbonate buffer. To measure polyclonal IgE, capture anti-IgE is used at 1:2000. Serum is diluted at 1:10 and detection anti-IgE is used at 1:1000; all dilutions are made in blocking buffer.

How to identify IgE receptor positive cells in whole blood samples

Forward/side scatter (FSC/SSC) plot of whole blood leukocytes reveals four distinct cell populations based on size and complexity (Figure 1A). Note that the FSC/SSC plot is generated using a log scale for SSC and a linear scale for FSC which allows for the signature separation of cells (Ganley-Leal et al., 2006). IgE-receptor bearing cells are present in each of the four populations. R1 contains FcεRII (CD23)+ B cells and FcεRI+ basophils (Figure 1B). It is important that FcεRII+ B cells are gated to measure cell-bound IgE to avoid quantifying IgE +CD27+ class-switched memory B cells, which will likely be elevated in patients with high IgE. R2 is the most heterogeneous cell population amongst blood samples and generally

contains monocytes, which are FcεRII+ and FcεRI+ (Figure 1B). It should be noted that two isoforms of FcεRI are found on human cells (Kinet, 1999). The tetrameric form has two common γ chains, an α chain and a β chain and the trimeric form lacks the β chain (Kinet, 1999). Monocytes express the trimeric form of FcεRI, therefore antibody must be directed against the α chain rather than the β chain on these cells. However, the α chain of FcεRI binds IgE (Kinet, 1999). Thus, some monoclonal antibodies directed against FcεRI α chain, may only detect the α chain if IgE is stripped from the cell surface.

R2 also contains mast cell precursors which are immature mast cells and generally FcεRI low and do not bind IgE in the blood. R3 is primarily neutrophils. Neutrophils have been reported to express IgE receptors (Gounni et al., 2001), though bound-IgE was generally low on neutrophils in our sample population. R4 is a relatively homogeneous population that contains eosinophils. Eosinophils express both FcεRI and FcεRII but activation levels can impact the level of expression of either of these IgE receptors. For example, under certain conditions, activated eosinophils may shed FcεRII and upregulate FcεRI (Mawhorter et al., 1996; Ganley-Leal et al., 2006).

Quantification of cell bound-IgE in blood

The level of blood cell-bound IgE is calculated using the percentage of FcεRII+IgE+ and FcεRI+IgE+ cells of total whole blood leukocytes and the Mean Fluorescence Intensity (MFI) of IgE on each cell population. The percentage of IgE+ cells within each IgE receptor-bearing cell population is multiplied by the MFI of IgE to generate an arbitrary Unit of IgE per cell type. The sums of Units will represent the amount of cell bound-IgE in blood.

Cell-bound IgE is measured on B cells, basophils and eosinophils for the following calculations. Figure 2A illustrates the measurement of cell-bound IgE on FcεRII+ B cells in R1. Since R1 is a mixed cell population, FcεRII+IgE+ cells must be first gated using a 2-color plot as shown in Fig. 2B. The percentage of FcεRII+IgE+ cells in R1 can be calculated using the 2-color plot as shown. To measure the relative level of IgE on this cell population, a histogram is generated using gate R5 (Fig. 2C). The histogram (Fig 2D) generated with gate R5 is overlaid with the isotype control using the same gate (anti-FcεRII + isotype control; not shown). The MFI of the isotype control is subtracted from the MFI of anti-IgE to calculate the level of IgE in this cell population. To determine how much B cell-bound IgE represents as a total of whole blood, the percentage of FcεRII+IgE+ in R1 must be multiple by the percentage represented by R1 of whole blood leukocytes (19% of R1 (Fig. 2B) × 24.1% of total blood leukocytes (Fig. 2A) = 4.6%). Verification of B cell expression of FcεRII can be performed by co-staining with anti-CD19.

Figure 2E illustrates the measurement of cell-bound IgE on FcεRI+ basophils in R1. FcεRI+IgE+ cells are first gated using a 2-color plot as shown in Fig. 2F. The percentage of FcεRI+IgE+ cells in R1 can be calculated using the 2-color plot as shown. To measure the relative level of IgE on this cell population, a histogram is generated using gate R6 (Fig. 2G). The histogram (Fig 2H) generated with gate R6 is overlaid with the isotype control using the same gate (anti-FcεRI + isotype control; not shown). However, the histogram generated with gate R6 contains considerable non-specific fluorescence that does not correspond with anti-IgE fluorescence on the 2-color plot (Fig. 2G). To reduce the non-specific fluorescence from the histogram, a gate (R7) is drawn around the remaining cell populations, R2, R3 and R4 (Fig. 2I) and is subtracted from R6 using the WinMDI function [-] to generate a new histogram (Fig. 2J). The newly generated histogram contains a peak that corresponds with the FcεRI+IgE+ population in Fig. 2G and is overlaid with the isotype control (Fig. 2J). The MFI of the isotype control is subtracted from the MFI of anti-IgE to calculate the level of IgE in this cell population. To determine how much basophil-bound IgE represents as a total of whole blood, the percentage of FcεRII+IgE+ in R1 must be multiple by the percentage represented by R1 of

whole blood leukocytes (1.7% of R1 (Fig. 2F) $\times$ 24.1% of total blood leukocytes (Fig. 2E) = 0.4%). Verification of basophils expression of Fc ϵ RI can be performed by co-staining with anti-CD63.

Since R4 is a relatively homogenous population, assessing the amount of cell-bound IgE does not require a 2-color plot (Ganley-Leal et al., 2006). The percentage of eosinophils of whole blood leukocytes can be accurately measured by using the R4 gate depicted in Figure 2K. A histogram can be generated directly from R4 and overlaid with the isotype control (Fig. 2L). The percentage of IgE+ eosinophils is calculated by measuring levels above the isotype control using the same markers for MFI measurements (Fig. 2L). To calculate the how much eosinophil-bound IgE represents as a total of whole blood, the percentage of IgE+ cells in R4 must be multiplied by the percentage represented by R4 of total blood whole blood leukocytes (4.0% of total $\times$ 76.7% IgE+ (Fig. 2L) = 3.1%). Verification of R4 containing eosinophils is performed by co-staining with anti-CD125.

The following are examples of cell-bound IgE calculations from two blood samples.

Sample 1: Typical blood sample with low cellular bound IgE:

Percentage of Fc ϵ R2+ B cells (1.53%) $\times$ percentage of IgE+ Fc ϵ R2+ B cells (27.3) = (0.41) $\times$ MFI of IgE (27) = 11.3 Units of B cell-bound IgE

Percentage of basophils (0.53%) $\times$ percentage of IgE+ basophils (83%) = (0.44) $\times$ MFI of IgE (123.8) = 54.5 Units of basophil-bound IgE.

Percentage of eosinophils (0.85%) $\times$ percentage of IgE+ eosinophils (46.5%) = (0.40%) $\times$ MFI of IgE (minus isotype control MFI) (55.0) = 21.7 Units of eosinophil-bound IgE

The sum of Units from each cell population: 21.7 + 11.3 + 54.5 = 87.5 Total units of cell-bound IgE.

Sample 2: Typical blood sample with high cellular bound IgE:

Percentage of Fc ϵ R2+ B cells (2.78%) $\times$ percentage of IgE+ Fc ϵ R2+ B cells (75.5%) = (2.1) $\times$ MFI of IgE (103) = 216.2 Units of B cell-bound IgE

Percentage of basophils (0.34%) $\times$ percentage of IgE+ basophils (89.6%) = (0.30) $\times$ MFI of IgE (2299.1) = 700.4 Units of basophils-bound IgE.

Percentage of eosinophils (9.22%) $\times$ percentage of IgE+ eosinophils (96.8%) = (8.92) $\times$ MFI of IgE (88.7) = 791.6 Units of eosinophil-bound IgE

The sum of Units from each cell population: 791.6 + 216.2 + 700.4 = 1708.2 Total units of cell-bound IgE.

Trouble shooting—In some instances, the FACS Lysis Buffer does not lyse the red blood cells (RBC) efficiently for performing flow cytometry on the whole blood. Inefficient lysing of RBC is evident when the cell suspension remains dark red following the washing step. The addition of one ml of PBS followed by vigorous pipeting up and down and an additional wash and resuspension in fresh PBS will generally result in enough RBC lysis to evaluate the cells by flow cytometry.

The use of isotype controls in flow cytometry has come into question (O'Gorman and Thomas, 1999). Nevertheless, we recommend the use of isotype controls for staining whole blood cells. The absence of isotype control might result in false intensities of cell-bound IgE, especially on granulocytes which express high levels of Fc γ . This concern is evident in Fig. 2L where eosinophils bind considerable amounts of isotype control which could vary greatly between samples. The use of Fc blocking reagent has not been tested on whole blood by our laboratory

and may be an alternative to isotype controls, though this would add an extra step to the assay described here.

Discrepancy between serum IgE levels and cell-bound IgE in whole blood

Plasma or serum IgE levels are considered to be reflective of systemic levels of IgE (Mitre et al., 2005); however because IgE receptor expression may change with allergy or parasite infection, ratios of cell-bound and free IgE molecules may be altered. To test whether our measures of cell-bound IgE correlate with serum IgE levels, concentrations of plasma polyclonal (total) IgE were plotted against measures of cell-bound IgE in 10 blood samples (Figure 3A). In general, levels of cell-bound IgE correlate well with free IgE in most blood samples. However, the concentration of cell-bound IgE has the potential to be extensive in the face of very low free serum IgE (Figure 3A; solid circle). Patients characterized by this profile may, for example, be high risk for developing anaphylaxis during a food challenge assay despite low serum IgE levels. In contrast, blood samples may have elevated serum IgE levels but low cell-bound IgE (Figure 3A; dotted circle). These results may be important for the interpretation of the role of IgE in host resistance to parasite infections, especially since IgE is thought to mediate most of its effector functions through IgE receptor + cells (Kinet, 1999).

Potential application of assay: Quantification of antigen-specific cell-bound IgE

A potential application in using this assay is to determine the relative level of cell-bound antigen-specific IgE to better understand risk factors of allergy or correlates of protective immunity. This demonstration makes the assumption that cell-bound and free IgE have the same ratio in the serum as on the cell, although specific microenvironments may contain cells that do not follow this paradigm. Measures of IgE are presented in multiple formats, including ng/ml of serum derived from ELISA, kilounits per liter derived from ImmunoCap (Quest Diagnostics), or more crude measures such as optical density (OD) and other colorimetric methods used in the field. Therefore, for demonstrative purposes, a basic ELISA is used to measure IgE and levels of IgE are presented as arbitrary units. Figure 3B demonstrates levels of polyclonal IgE and dust-mite specific IgE in a random sampling. To approximate levels of antigen-specific cell-bound IgE, ratios of the arbitrary unit of dust mite-specific IgE to polyclonal IgE are determined.

For example, Sample 1 above has 220 units of dust mite-specific IgE: 320 units of polyclonal IgE = (0.69). The ratio (0.69) is multiplied by the Units of cell-bound IgE in Sample 1 (87.5) which generates the Units of antigen-specific cell-bound IgE (60.3).

Sample 2 has 20 units of dust-mite specific IgE: 410 Units of polyclonal IgE = (0.05). The ratio (0.05) is multiplied by the Units of cell bound IgE in Sample 2 (1708.2) which generates the Units of antigen-specific cell-bound IgE (85.4).

Interestingly, both Samples have similar levels of cell-bound dust mite-specific IgE despite the drastic differences in the level of cell-bound IgE and plasma dust-mite specific IgE. This readout may indicate a similar level of allergic risk for a specific antigen further illustrating the importance of measuring cell-bound IgE (Mitre et al., 2005).

Conclusions

We present a simple method for a better quantification of somatic IgE levels by combining cell-bound levels with free IgE concentrations. Although this assay is not inclusive of tissue mast cell-bound IgE, including measures of cell-bound IgE, especially antigen-specific cell bound IgE, in human studies of helminthiasis may lead to a better understanding of the elusive role this molecule has in host protection. Furthermore, because this assay requires a small

amount of blood (less than 2 mls), it could represent a useful tool in predicting severe food allergies, especially in children. Other potential applications include using fluorescently-labeled antigens/allergens to detect antigen-specific cell-bound IgE on blood cells. Quantification of unengaged IgE receptors in blood from patients on anti-IgE Fc may also be a valuable in determining the efficacy of this treatment. Validation in the clinic and in the field and uniformity in the presentation of Units of IgE will likely provide a useful, simple tool for better IgE quantification and an understanding of the role of IgE in host immunity.

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Figure 1. Identifying IgE receptor-positive cells in whole blood samples

A. Electronic separation of whole blood leukocytes by linear Forward scatter (FSC) and log Side scatter (SSC) plots. B. Four distinct cell populations are revealed by regions (R)1-R4. C. IgE receptor expression in regions R1, R2, and R4. IgE-receptor positive cells in R1 are CD19⁺ B cells (FcεRII⁺) and basophils (FcεRI⁺). R2 contains monocytes (FcεRI⁺ and FcεRII⁺) and mast cell precursors (FcεRI^{low}). R3 contains neutrophils which are generally IgE receptor negative (not shown). R4 contains eosinophils which are FcεRI⁺ and FcεRII⁺ depending upon their activation status. D. Cells expressing IgE receptors in R1, R2, and R4 circulate with cell-bound IgE. All gates were drawn with IgE receptor + isotype control.

Measurement of IgE bound to FcεRII+ B cells

Measurement of IgE bound to FcεRI+ Basophils

Measurement of IgE bound to FcεRII/FcεRI+ eosinophils

Figure 2. Measuring cell-bound IgE levels in whole blood

A. Measurement of IgE on FcεRII+ B cells. The first step in measuring IgE bound to B cells is to gate on R1 which contains a mix of lymphocytes and basophils. B. The density plot displaying FcεRII and IgE in R1 allows for the calculation of FcεRII+ cells which are IgE+ (19.0%). C. FcεRII+/IgE+ cells are gated as R5 and displayed as a histogram to display the level of IgE (D). The isotype control is overlaid and the geometric mean fluorescence intensity is measured. The MFI of IgE on FcεRII+ B cells is calculated by subtracting the isotype control MFI from the anti-IgE MFI (150.4-16.0=134.4). E. Measurement of IgE on FcεRI+ basophils. The first step in measuring IgE bound to basophils is to gate on R1. F. The density plot displaying FcεRI and IgE in R1 allows for the calculation of FcεRI+ cells which are IgE+ (1.7%). G. FcεRI+/IgE+ cells are gated as R6 and displayed as a histogram to display the level of IgE (H). However, despite gating on R6, a significant amount of non-specific signal is evident on the histogram which likely originates from the remaining granulocytes in blood. I. A gate (R7) is drawn around R2, R3 and R4 (see Fig. 1). J. A new histogram is generated by gating positively on R6, using the [+] function in WinMDI and negatively on R7 using the [-] function. The histogram now contains the IgE high cells displayed in R6 (G) and is overlaid with an isotype control. The MFI of IgE on FcεRI+ basophils is calculated by subtracting the isotype control MFI from the anti-IgE MFI (2447.1-17.1=2430). K. Measurement of IgE on FcεRII+/FcεRI+ eosinophils. The first step in measuring IgE bound to eosinophils is to gate on R4. Because R4 is a relatively pure population of eosinophils, histograms can be directly

generated from R4 and overlaid with the isotype control (L). The percentage of IgE+ eosinophils can be inferred from the histogram (76.7%+). The MFI of IgE on FcεRII+/FcεRI + eosinophils is calculated by subtracting the isotype control MFI from the anti-IgE MFI (89.1-31.3= 57.8).

Figure 3. Relationship between levels of free IgE and cell-bound IgE

A. Levels of polyclonal IgE (ng/ml) were plotted against Units of cell-bound IgE (n=10).

Dotted circle demonstrates a sample with high serum levels of IgE and low cell-bound IgE.

Solid circle depicts a sample which has extensive cell-bound IgE but low free IgE molecules in the plasma.

B. Sample IgE levels of polyclonal IgE vs antigen-specific (dust mite) IgE.

Arbitrary units were generated by multiplying the OD by 1000.