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An ELISA-based method for measurement of food-specific IgE antibody in mouse serum: an alternative to the passive cutaneous anaphylaxis assay

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Abstract

Passive cutaneous anaphylaxis (PCA) assay has been a gold standard method to measure allergen-specific IgE antibody (ASIgE Ab) levels in allergy mouse models. Many factors including stringent guidelines for laboratory animal use make PCA a difficult choice. Therefore, alternative methods are needed that can be readily applied for measurement of specific IgE antibody levels in mouse serum. Herein we describe a novel ELISA-based method that is more sensitive in comparison to PCA, IgE isotype-specific (because it has little cross-reactivity with IgG1 or IgG2a isotype) and highly reproducible (< 10% inter- or intra-assay variation). Furthermore, we demonstrate the utility of this assay to measure specific IgE Ab against a variety of food extracts including chicken egg, peanut, almond, filbert/hazelnut and sweet potato. These findings are of particular interest to those who are seeking (i) to measure food-extract-specific IgE antibody in animal models and (ii) an alternative to the animal-based PCA method to measure mouse IgE antibodies.
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1. Introduction

Allergen-specific IgE antibody (ASIgE Ab) production is a central event in the pathogenesis of atopic disorders that include allergic asthma, rhinitis, dermatitis, conjunctivitis, food and drug allergies, and anaphylaxis (Babu et al., 2001; Platts-Mills, 2001; Wilson et al., 2002). Consequently, the presence of elevated levels of specific IgE Ab in the serum is a

Abbreviations: ASIgE, allergen-specific IgE; Ig, immunoglobulin; OD, optical density; ELISA, enzyme-linked immunosorbent assay; PCA, passive cutaneous anaphylaxis assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; S.D., standard deviation; S.E., standard error; RAST, radioallegrosorbent test.

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diagnostic factor for immediate hypersensitivity response to environmental antigens in humans and animal allergy models (Perelmutter, 2001). Therefore, accurate, reliable and user-friendly methods are needed to measure serum levels of allergen-specific IgE antibodies.

Passive cutaneous assay (PCA) performed in mice or rats has been widely used to measure allergen-specific IgE antibody levels in animal models for half a century (Gaveriaux et al., 1986; Gieni et al., 1993; Gaboriau-Routhiau and Moreau, 1997; Chen et al., 1998) The PCA method has the advantage of measuring not only the biologically active IgE Ab but also the consequence of allergen/IgE interaction leading to the inflammatory mediator release from mast cells and the clinical expression of cutaneous anaphylaxis. However, many factors including stringent guidelines for laboratory animal use, its labor-intensive nature and the capacity of murine IgG1 to trigger PCA responses limit the utility of this assay. Therefore, alternative in vitro methods that are inexpensive, easy to perform and comparable in sensitivity to PCA are needed.

A number of ELISA-based methods have been described for measuring IgE Ab specific to individual purified proteins such as ovalbumin, milk proteins (casein, beta-lactoglobulin), peanut major allergens (Ara h1, Ara h2), or haptens (Fox et al., 1976; Hill and Liu, 1981; Hall and Rittenberg, 1985; Gaveriaux et al., 1986; Harada et al., 1987; Hanashiro et al., 1996; Li et al., 1999, 2000; Adel-Patient et al., 2000; Bilenki et al., 2002). However, we are not aware of methods available for measuring whole food-extract-specific IgE antibody in mouse serum. In an effort to fill this need, here we describe an ELISA-based method for food-extract-specific IgE Ab detection that is comparable in sensitivity to PCA assay. Furthermore, we demonstrate the utility of this method by performing specific IgE Ab detection using a variety of food extracts including chicken egg, peanut, almond, filbert/hazelnut and sweet potato.

2. Materials and methods

2.1. Materials

The following materials were purchased from sources as indicated in parenthesis: food extracts (Greer Labs, Lenoir, NC, USA); biotin-labeled anti-mouse IgG1 (Southern Biotech, Birmingham, AL), anti-mouse IgG2a (Southern Biotech) and anti-mouse IgE antibody (Serotec, Raleigh, NC; BD PharMingen, San Deigo, CA, USA); Ig isotype standards (Southern Biotech); *p*-nitro-phenyl phosphate (PNPP, Sigma, St Louis, MO, USA); streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); protein-G Sepharose (Pharmacia Biotech); ovalbumin (ICN Bio-Medicals, Montreal).

2.2. Mice

All mice were purchased from Jackson Lab (Bar Harbor, ME, USA). Only adult animals (6–7 weeks of age) were used in the study. All animal procedures used were in accordance with the Michigan State University policies.

2.3. Animal immunization and serum collection

Various standardized food protein extracts purchased from Greer labs were sterilized by filtration and total protein content was determined by Lowry's method. A group of adult mice ($n=5$) received intraperitoneal injection of 100 μg of protein extract from peanut, almond, filbert/hazelnut, walnut and chicken egg plus alum (2.5 mg/mouse) as an adjuvant. Another group ($n=5$) received 100 μg of protein extract from chicken egg, wheat, soy, coffee and sweet potato plus alum (2.5 mg/mouse). Control mice ($n=3$) received sterile saline injections. All mice were bled on day 11 after first injection. Animals received booster injection and bled on days 7, 15, 32 and 62 after the booster injection. The serum was used in antibody estimations.

2.4. Food-specific IgE, IgG1 and IgG2a antibody measurement

Enzyme-linked immunosorbent assay (ELISA) was optimized for each of the food extracts taking into account the background activity (i.e., all reagents added except for the sample). All reagents were used at a final volume of 50 μl/well except for blocking buffer that was used at 75 μl/well. Washing was done with 200 μl/well using an automatic ELISA washer (Dynex Technologies, Ultra-wash Plus). Each food extract was analyzed for protein content by Lowry's

method and then used in ELISA for coating at concentrations ranging from 10 to 5000 μg/ml. Briefly, ELISA plates (96-well EIA/RIA plate, 96-well easy wash™, high binding, Corning, New York) were coated with food extracts diluted in carbonate buffer (0.05 M, pH 9.6) and incubated at 4 °C, overnight. Unbound extract was discarded and the plates were blocked (0.17% BSA/PBS) at 37 °C. For peanut IgE assay, blocking was performed with 5% gelatin. After washing (0.05% Tween 20 in PBS), serum samples were added at various twofold dilutions from 1:20 to 1:640 or in some experiments at 1:30 to 1:61,440 in dilution buffer (0.085% BSA, 0.05% Tween 20 in PBS). Following incubation, plates were washed and a biotin-labeled anti-mouse IgG1 or IgG2a or IgE antibody added. After incubation, plates were washed and streptavidin alkaline phosphatase conjugate was added at 1:4000 (in dilution buffer). Subsequently, plates were washed and *p*-nitro-phenyl phosphate (PNPP) substrate added (1 tablet per 5 ml substrate buffer, according to manufacturer's instruction; Sigma). Reactions were allowed to develop at room temperature in the dark and absorbance measured in a microplate reader using dual mode of wavelength at 405 nm (peak) minus 690 nm (background) (Microplate ELISA Reader, SoftMax program, Molecular Devices). According to manufacturer's instructions, dual mode provides relatively better measurements since it adjusts the reading for background interference (Personal communication, Technical Services, Molecular Devices). All plates included negative controls (no mouse serum sample background and no antigen coating control) and a positive internal control (a reference mouse serum sample containing known levels of ovalbumin-specific IgE antibody; a kind gift from Prof. Kent HayGlass, The University of Manitoba). All samples were analyzed for a minimum of two to three times.

2.5. Determination of assay sensitivity and serum antibody titer

The assay sensitivity was defined as the background optical density from wells to which all reagents but no mouse serum sample had been added, +3 S.D.

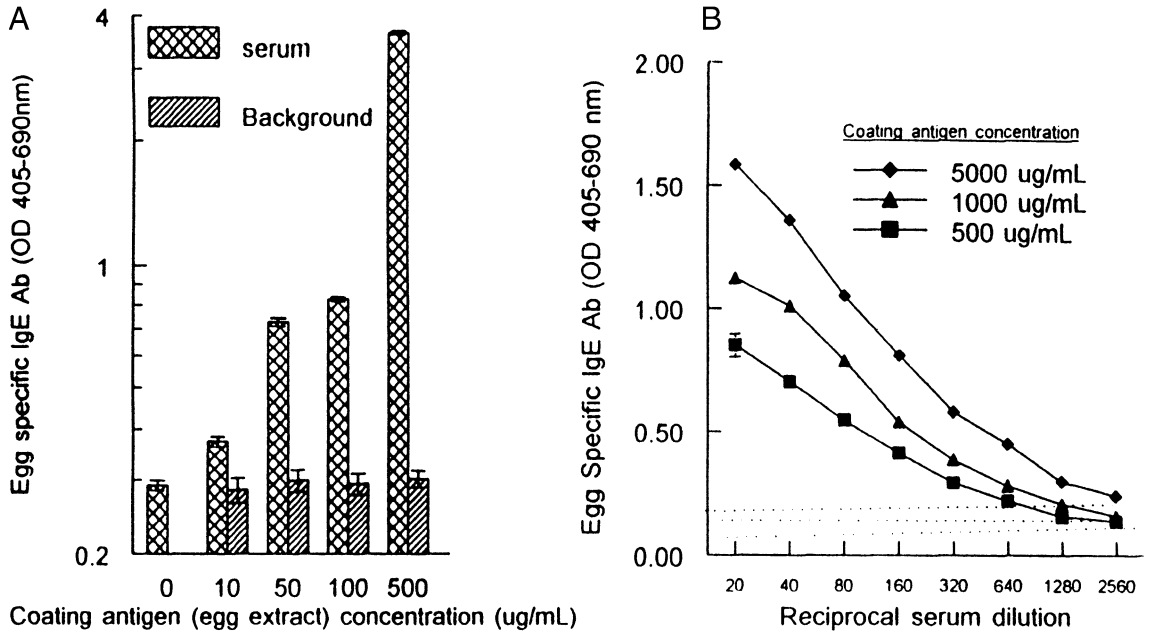


Fig. 1. (A, B) Impact of coating antigen concentration on assay sensitivity. (A) Wells were coated with various amounts of egg protein extract (10–500 μg/ml) and egg-specific IgE Ab in the serum obtained from egg-sensitized mice was measured after overnight (A) or ~2 h developing time (B). For A, serum was used at 1:10 dilution. The same serum sample was used at indicated dilutions in B. Data represent mean ± S.E. of duplicates. Broken lines indicate background OD at three different coating Ag concentrations.

Antibody titer was defined as the reciprocal of the serum dilution that closely matched this value.

2.6. Passive cutaneous anaphylaxis assay

Anti-ovalbumin IgE levels were determined by 48-h PCA assay in female Sprague–Dawley rats as previously described (HayGlass and Strejan, 1984). Means of duplicate or triplicate analyses are presented.

3. Results

3.1. Impact of coating antigen concentration on food-specific IgE antibody detection

In order to determine the optimal coating antigen concentration for IgE antibody detection, initially, an ELISA was set up using various amounts of egg extract. As evident from the results, a coating antigen concentration of 500 μg/ml yielded maximal absorbance (OD). (Fig. 1A). However, the assay took 24-h incubation for complete color development. Furthermore, the OD was off the scale implying off accuracy and off precision. We rationalized that increasing the concentration of coating antigen might reduce the developing time it takes to get a reasonably good assay with a good detection window. Therefore, we used 2- to 10-fold higher antigen amounts for coating. As evident (Fig. 1B), a combination of higher coating antigen concentration (at 5000 μg/ml instead of 500 μg/ml) and a lower developing time of 2 h (instead of 24 h) provided an assay with a reasonably good window of detection. Furthermore, the background activity was not significantly enhanced with higher coating antigen

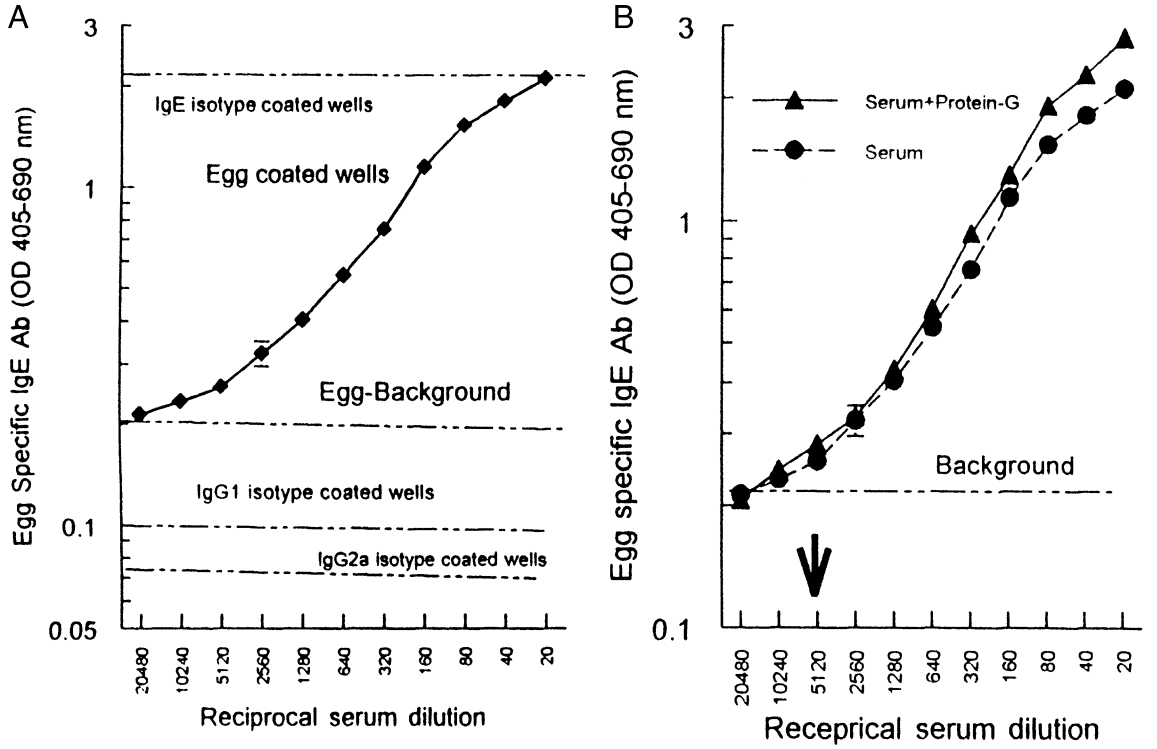


Fig. 2. (A, B) Immunoglobulin (Ig) epsilon isotype specificity of the assay. (A) Wells were coated with either the egg extract or Ig isotype controls (IgG1 at 500 ng/ml, IgG2a at 500 ng/ml, IgE at 250 ng/ml), followed by serum (from egg-sensitized mice) addition to egg-coated wells only and developed with anti-mouse IgE antibody. (B) Serum from egg-sensitized mice was pretreated with 50% slurry of protein-G Sepharose following manufacturer's instructions (Pharmacia Biotech) and then subjected to egg-specific IgE ELISA. Data represent mean ± S.E. of duplicates or triplicates. Arrow indicates the egg-specific IgE Ab titer.

concentrations. Thus, coating food extract at a protein concentration of 5000 μg/ml and a developing time of 1–3 h was used in all subsequent assays. This data suggests that the antigen did not bind to the microplate well in sufficient quantities except at high concentration.

3.2. Immunoglobulin epsilon isotype specificity of the assay

We tested the isotype specificity of the assay by two different ways: (i) first, we compared the isotype specificity of the detection antibody. In this experi-

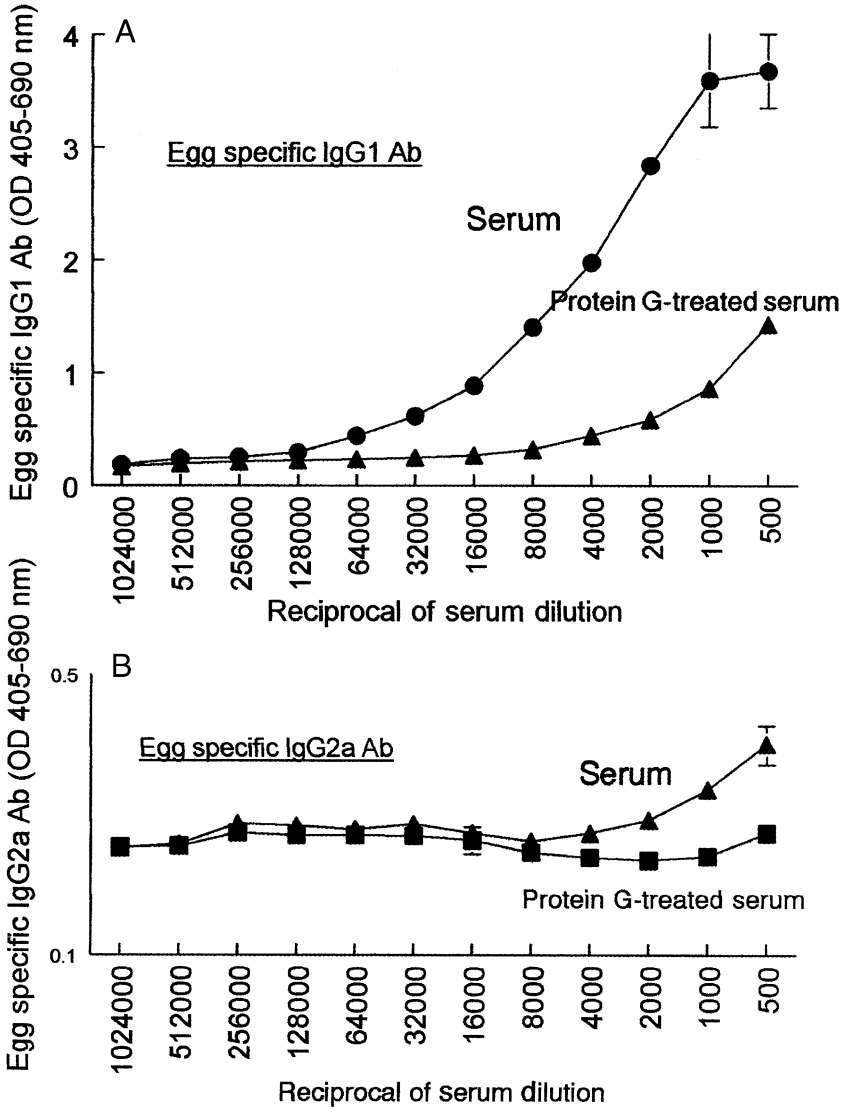


Fig. 3. (A, B) Impact of protein-G treatment on egg-specific IgG1 and IgG2a antibody levels in serum from mice sensitized with egg. Wells were coated with the egg extract followed by addition of serum with or without protein-G treatment (50% slurry), following manufacturer's instructions (Pharmacia Biotech) and developed with biotin-labeled anti-mouse IgG1 (A) or IgG2a (B) antibody. Serum sample was identical to the one used in Fig. 2. Data represent mean ± S.E. of duplicates or triplicates.

ment, we coated wells with mouse IgE (at 250 ng/ml), IgG1 (at 500 ng/ml) and IgG2a (at 500 ng/ml) Ig isotype standards or egg, and then added serum samples from egg-sensitive mice to egg-coated wells and buffer to isotype standard-coated wells. All wells were developed with a biotin-conjugated anti-IgE antibody. As evident from the results (Fig. 2A), biotin antibody detected egg-specific IgE antibody. Furthermore, biotin antibody reacted with IgE but not with IgG1 or IgG2a isotype standards verifying the epsilon isotype specificity of the assay. (ii) Second, we depleted IgG1 and IgG2a from mouse serum by treating it with protein-G Sepharose following manufacturer's instructions (Pharmacia Biotech) and tested its impact on IgE detection by the assay (Fig. 2B). Removal of IgG1 and IgG2a from mouse serum indeed enhanced the detection window of the assay (i.e., peak signal minus the background OD) although it had no significant impact on the assay sensitivity. A control experiment was performed to make sure that protein-G treatment indeed removed most of the egg-specific IgG1 and IgG2a from mouse serum (Fig. 3).

3.3. Comparison between ELISA and PCA assays

In order to compare the relative sensitivity of ELISA vs. PCA to measure mouse IgE antibodies, we measured ovalbumin-specific IgE Ab titer of serum sample by both methods. As evident (Fig. 4), ELISA titer of the sample (15,360) was about two titers higher compared to that of PCA titer (3900). These data suggest that the ELISA method described here might be a suitable alternative to PCA method for measuring mouse IgE antibodies.

3.4. Reproducibility of the ELISA method

We examined the inter-assay and intra-assay variation of this assay when performed by two different individuals. As evident from the data, coefficient of variation in all cases was $< 10\%$ (Fig. 5).

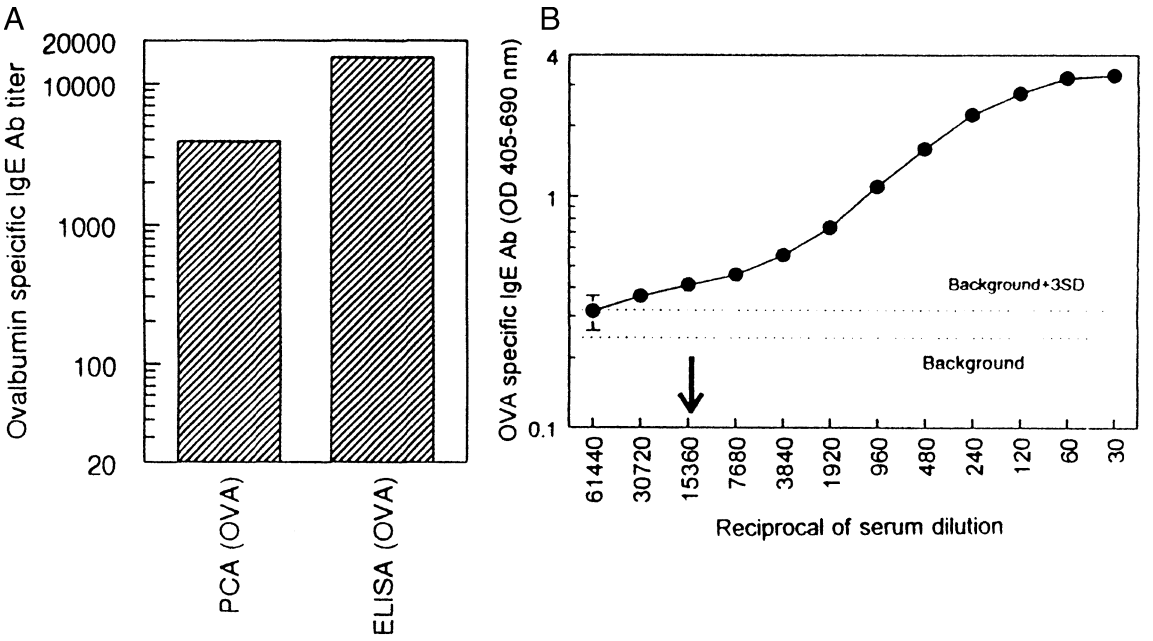


Fig. 4. (A, B) Comparison of assay sensitivity: ELISA vs. PCA. (A) An identical mouse serum sample was subjected to ELISA and PCA and ovalbumin (OVA)-specific IgE antibody titer determined. (B). This figure demonstrates the method used to deduce ovalbumin-specific IgE Ab titer by the ELISA method that is shown in A. Data from triplicate experiments is presented (mean ± S.E.). Arrow indicates the OVA-specific IgE Ab titer.

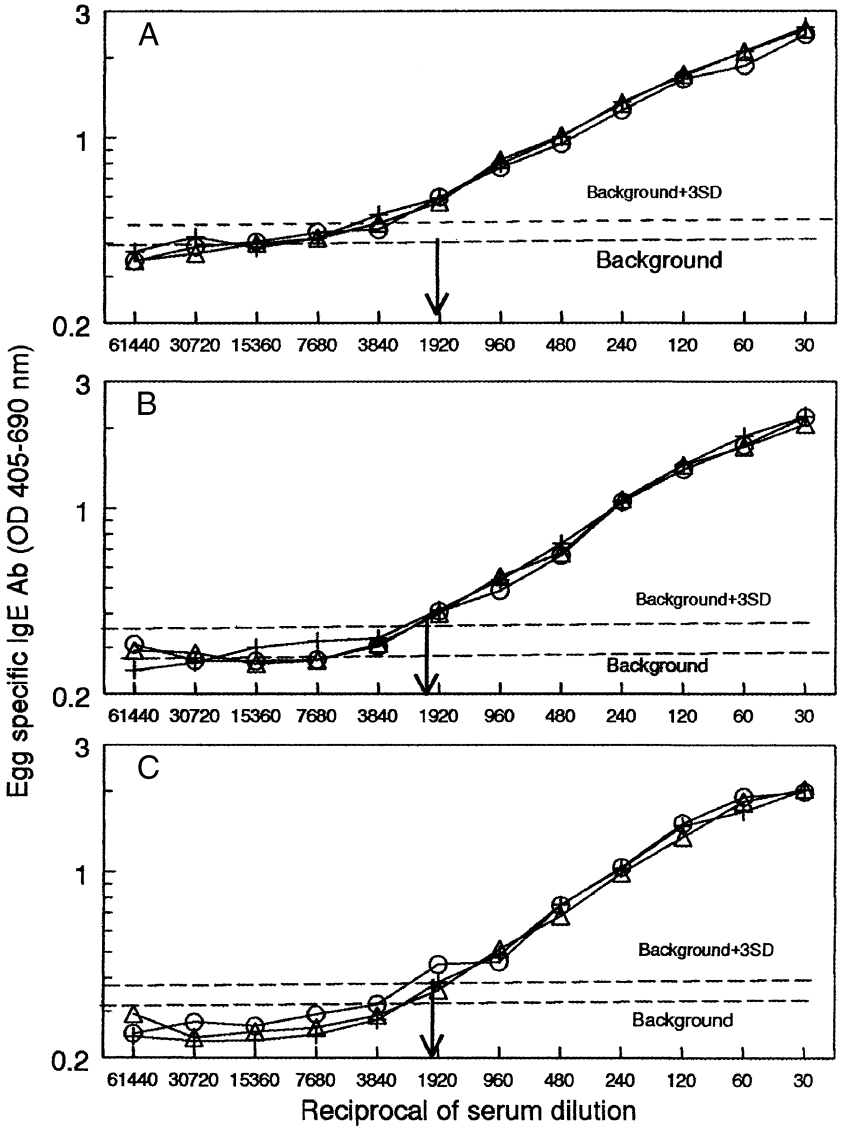


Fig. 5. (A–C) Inter- and intra-assay variation of the ELISA method. (A) This figure illustrates three sets of data obtained by screening an identical sample on the same ELISA plate (intra-assay variation). (B, C) Data shown illustrates three sets of data obtained by screening an identical sample on different ELISA plates on different days by two different persons (inter-assay variation). Data represent mean ± S.E. of triplicates. Arrow indicates the egg-specific IgE Ab titer.

3.5. Application of the assay to measure food-specific IgE antibody levels in mouse serum

We tested the potential utility of the ELISA method to measure food-extract-specific IgE antibody against a variety of food types using food-sensitized mouse serum. As evident (Fig. 6), this method was useful to measure specific IgE Ab levels using extracts from chicken egg, peanut, almond, hazelnut and sweet potato. The average background activity (OD) for various food types at 2-h reading was as follows: egg ≤ 0.3, peanut extract, ≤ 0.26, filbert/

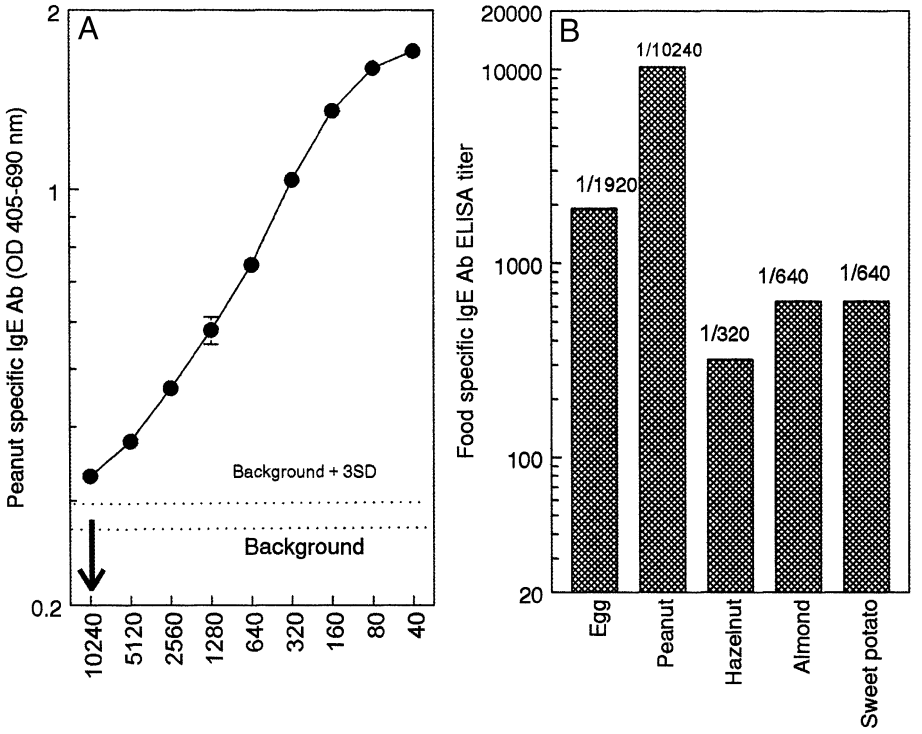


Fig. 6. Applications of the ELISA method to measure food-extract-specific IgE Ab in mouse serum. (A) Data shown in this figure illustrates the utility of this method to determine peanut-specific IgE antibody titers in the serum of mice sensitized with peanut. Reciprocal of the serum dilution with OD that closely matched the background OD + 3 S.D. was defined as the IgE antibody titer of the serum sample (shown as an arrow). (B) Similar method as shown in A was used to deduce IgE titers against other food types using serum from mice sensitized with various food types. Data represent mean ± S.E.

hazelnut ≤ 0.38, almond ≤ 0.3 and sweet potato ≤ 0.06.

4. Discussion

We have described an ELISA-based method for measurement of food-specific IgE antibody in mouse serum using a variety of food types. The data presented demonstrates that the ELISA-based method is more sensitive than PCA, isotype-specific (because it has little cross-reactivity with IgG1 and IgG2a) and highly reproducible ($< 10\%$ inter-assay and intra-assay variation). We are not aware of any previously described ELISA methods for food-extract-specific IgE antibody detection in mouse serum.

Three methods have been widely used to measure allergen-specific IgE antibody in the serum samples, namely PCA, RAST and ELISA, although several other methods (e.g., in vitro histamine release assay, fluorometric reverse (IgE-capture) ELISA, resetting, etc.) have been described in the literature (Fox et al., 1976; Hill and Liu, 1981; Bohn and Konig, 1985; Hall and Rittenberg, 1985; Gaveriaux et al., 1986; Sedgwick and Holt, 1986; Bozelka et al., 1987; Harada et al., 1987; Sakaguchi et al., 1989; Peng et al., 1993; Doekes et al., 1996; Hanashiro et al., 1996; Adel-Patient et al., 2000; Cruz et al., 2000; Perelmutter, 2001; Bilenki et al., 2002). PCA has several advantages including (i) high sensitivity, (ii) high specificity (if challenged 48 h post sensitization) and (iii) measurement of biological consequence of allergen–IgE interaction. However, it suffers from many practical disadvantages as follows: (i) high cost; (ii) animal use that is labor-intensive; and (ii) concerns about animal welfare. The RAST is routinely used clinically for measurement of specific IgE Ab in the human serum (Sakaguchi et al., 1989; Perelmutter, 2001; Sicherer,

2001; Wilson et al., 2002). Although it is applicable to a mouse system, its utility is limited by the use of radioisotopes and low level of precision (i.e., 1+, 2+ as readouts). In contrast, this ELISA method addresses the many shortcomings of PCA and RAST methods.

Currently there are few ELISA-based methods available for determination of specific IgE Ab against purified proteins derived from foods including ovalbumin (chicken egg-derived), milk proteins (casein, beta-lactoglobulin) and Ara h1, Ara h2 (peanut-derived) (Li et al., 1999, 2000; Adel-Patient et al., 2000; Bilenki et al., 2002). While these methods are very useful for individual purified proteins, their suitability for whole food extracts from different sources has not been demonstrated. Furthermore, we are not aware of previous reports on measuring specific IgE antibody in mouse serum for either whole extract or purified proteins from almond, hazelnut and sweet potato.

Some of the specific IgE antibody measuring ELISA methods employ use of protein-G to remove competing IgG1 from the serum (Bilenki et al., 2002). We found that depleting IgG1 and IgG2a from the mouse serum enhanced detection window of the assay (Fig. 3), suggesting competition between specific IgG1/IgG2a and IgE antibody for common epitopes. However, the sensitivity of the assay apparently was not affected by this competition.

In the present method, we used alkaline phosphatase-dependent substrate (*p*-nitro-phenyl phosphate) for color development. It may be possible to further enhance the sensitivity of this method using alkaline phosphatase-dependent fluorescence substrates (e.g., 4-methyl-umbelliferylphosphate).

In the present study, the ELISA method described here was tested for general applicability using five different food types. We noticed that peanuts consistently exhibited higher background activity when BSA was used as a blocking agent. In contrast, use of 5% gelatin as a blocking agent dramatically reduced background and increased assay sensitivity. We have also found that the method described here is suitable for a number of other food types (data not shown). We conclude that the method described here may be applied to detect IgE antibodies in mouse serum against a variety of food types—not necessarily *all* food types.

In summary, we have described an ELISA-based assay that would be very useful to those who are seeking (i) to measure food-extract-specific IgE antibody in mouse models of food allergy and (ii) an alternative to animal-based PCA method to measure mouse IgE antibodies.

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