

Enhancement of murine IgE antibody detection by IgG removal

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Received 25 November 2002; received in revised form 5 August 2003; accepted 5 August 2003

Abstract

Rationale: Although animal models for the study of allergic reactions are desirable, the use of mice has been hindered by the lack of sufficiently sensitive in vitro immunoglobulin epsilon (IgE) antibody assays. The aim of this study was to enhance IgE antibody measurements by immunoglobulin gamma (IgG) depletion. **Methods:** Seven- to eight-week-old female mice of four strains (C3H/HeJ, CBA/J, C57Bl/6J, and Balb/c) were immunized (20 mice/group) with shrimp or peanut extracts using Al(OH)₃ as adjuvant. Following immunization, animals were sacrificed by exsanguination and the sera of each group pooled. Initial measurements of IgE antibody levels by enzyme-linked immunosorbent assay (ELISA) were relatively low; IgG and IgE reactivity patterns by immunoblot were similar. Thus, sera from shrimp or peanut immunized mice were depleted of IgG (absorbed 3–6 times with immobilized protein G) and then tested for IgE antibody to shrimp or peanut allergen. **Results:** A 3- to 5-fold increase in IgE antibody reactivity as measured by ELISA was demonstrated when >80–90% of the IgG was removed. This increase in detection of allergen-specific IgE occurred in sera from all mouse strains and to all allergens tested. In addition, reactivity of IgE antibodies to peanut or shrimp allergens by immunoblot increased visually approximately 4- to 10-fold. **Conclusions:** These studies indicate that allergen-specific IgG antibodies, which may be in more than 100-fold excess to IgE antibodies, interferes with detection of allergen-specific IgE, probably by competitive binding to allergenic epitopes. Substantial depletion of IgG antibodies (>80%) result in a significant increase in the sensitivity of the antibody measurements.

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Keywords: IgE antibody detection; IgG depletion; IgE anti-shrimp; IgE anti-peanut

1. Introduction

Due to current concerns about the allergenicity of novel proteins expressed in major food crops, there is renewed interest in development of animal models of

food allergy and to assess a protein's allergenicity (Helm, 2002; Atherton et al., 2002; Knippels and Penninks, 2002; Buchanan and Frick, 2002). Of the animal species studied for immunoglobulin epsilon (IgE) antibody production, the mouse is a particularly attractive one since it has been documented for many years to produce IgE (Lehrer, 1976), has a well-characterized immune system with many reagents available for its study, and provides a variety of inbred strains for study. However, measurement of allergen-specific IgE antibodies from immunized mice yields low or negative values. The current investigation

Abbreviations: IgE, immunoglobulin epsilon; IgG, immunoglobulin gamma; ELISA, enzyme-linked immunosorbent assay; MWCO, molecular weight cut off.

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tested the effect of the removal of immunoglobulin gamma (IgG) antibodies on the sensitivity of allergen-specific IgE antibody measurements.

2. Materials and methods

2.1. Allergen extracts

Peanut extract: 20 g of flour from roasted Virginia-type and runner-type peanuts (gift of Golden Peanut, Alpharetta, GA) were homogenized in 200 ml PBS (pH 7.2) in five 2 s bursts in a Waring® blender. The slurry was stirred at 4 °C for 4 h, centrifuged at $100,000 \times g$ for 30 min, and the supernatant collected, dry weight and protein concentration determined by the [Bradford method \(1976\)](#) using a commercial kit (Pierce, Rockford, IL), aliquoted, frozen, and stored at -20 °C.

Shrimp extract: Brown shrimp (*Penaeus aztecus*) were boiled for 15 min in ion-depleted water. The shrimp meat was homogenized for 1 min in PBS (pH 7.2) with a Waring® blender, and stirred overnight at 4 °C. The extract was centrifuged at $40,000 \times g$ and the supernatant concentrated using an Amicon YM-1 (Bedford, MA) with a molecular weight cut-off (MWCO) of 1000 Da. The concentrate was recentrifuged at $80,000 \times g$, the supernatant harvested, protein concentration determined, aliquoted, frozen, and stored at -20 °C.

2.2. Sensitization of mice

Female mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 3–5 weeks of age. For peanut immunization, 3-week-old mice were kept on peanut/soy free mouse chow (Harlan Teklab, Madison, WI) prior to and during sensitization. For shrimp immunization, 5-week-old mice were obtained and kept on normal mouse feed (Purina, St. Louis, MO). At 7–8 weeks of age, four mouse strains (Balb/c, C3H/HeJ, C57Bl/6J, CBA/J) were sensitized by 0.2 ml intraperitoneal (i.p.) injections with 10 µg allergen absorbed to 1 mg $\text{Al}(\text{OH})_3$ (Sigma, St. Louis, MO), suspended in 0.9% NaCl. The injections were repeated weekly for 5 weeks. All animals were sacrificed by exsanguination 1 week following the last injection of antigen, and 20 individual sera of each mouse strain were pooled.

2.3. Absorption of IgG antibody with protein G

For removal of IgG, equal volumes of protein G beads (DynaL Biotech, Lake Success, NY) and mouse serum (diluted or undiluted) were mixed and incubated at room temperature for 30 min. The sample tube is then placed on a magnet to collect the beads at the tube wall and the supernatant serum removed and assayed for allergen-specific IgG. The beads are regenerated with elution buffer (0.1 M sodium citrate, pH 2.8) followed by 2 washes with 0.1 M sodium phosphate, pH 6.0, and the process repeated until approximately 80–90% of IgG antibodies was removed from the serum. The serum volume was measured prior to and following absorption and it was shown that approximately 80% was recovered following three to five absorptions. Although we cannot exclude some dilution occurring, it is unlikely, and if it occurred, it was minimal.

2.4. ELISA for allergen-specific mouse IgE and IgG antibodies

EIA/RIA 96-well, high-binding plates (Corning, Corning, NY) were coated with 100 µl containing 5 µg allergen extract or 2 µg/ml anti-IgE (Pharmingen, San Diego, CA) for the standard curve in PBS, pH 7.2 overnight at 4 °C, or for 1 h at 37 °C. After washing five times with PBS-Tween, blocking with PBS pH 7.2 containing 0.05% Tween 20 and 1% BSA (PBS-T+BSA) at RT for 1 h, the plates were incubated with diluted (1:10–1:160) mouse serum or 1–200 ng/ml IgE (Pharmingen) for the standard curve for 1 h at RT. After 5 washes, the plates were incubated for 1 h with biotinylated anti-mouse IgE R35-118, at 1:250 (Pharmingen) or with biotinylated anti-mouse IgE, Clone 23G3 diluted to 1:4000 (Southern Biotechnology Associates, Birmingham, AL) for 1 h, and washed five times with PBS-T. After incubation for 1 h with horseradish peroxidase (HRP)-labeled streptavidin diluted to 1:1000, (Pharmingen, San Diego, CA) followed by 5 washes, the IgE antibody binding was visualized using 1% (w/v) TMB and 30% (w/w) H_2O_2 in 100 mM sodium acetate buffer pH to 6.0 as substrate. After incubation for exactly 3 min (in the dark), the reaction was stopped with 1 M 85% *o*-phosphoric acid (Fisher, Fair Lawn, NJ). For the detection of allergen-specific IgG antibodies, a sim-

Table 1

Shrimp-specific IgE antibody levels in unabsorbed and IgG-depleted (absorbed) mouse sera

Mouse strain	Unabsorbed		Absorbed		IgG removal (%)	Increased IgE detection
	IgG [$\mu\text{g/ml}$]	IgE [$\mu\text{g/ml}$]	IgG [$\mu\text{g/ml}$]	IgE [$\mu\text{g/ml}$]		
C ₃ H/HeJ	794.0	4.4	23.3	18.5	97.1	4.2 $\times$
CBA/J	1202.9	2.0	71.5	8.5	94.1	4.3 $\times$
Balb/c	2631.9	0.023	59.9	0.333	97.7	14.5 $\times$
C57Bl/6J	17.7	0.3	<1.5	0.8	>91.5	2.7 $\times$

ilar procedure was employed, diluting mouse sera 1:20,000–1:160,000, and a peroxidase-labeled goat anti-mouse IgG 1:2000 (Kirkegaard and Perry Laboratories, Gaithersburg, MD) to detect IgG antibody reactivities. For the IgG standard curve, an anti-mouse IgG antibody (Sigma) at 2 $\mu\text{g/ml}$ and mouse IgG (Sigma) in the range from 1 to 200 ng was used as capture antibody and standard, respectively (Li et al., 2000).

2.5. SDS-PAGE and immunoblotting

Immunoblotting was performed as reported previously (Ayuso et al., 1999). Briefly, SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970) in 0.5 mm thick gels. A 1.5-cm-high stacking gel (5%T, 1.5%C) was poured on top of a 6.5 cm high separating gel (12.5%T, 1.5%C). After separation (12.5 μg protein/cm), proteins were transferred onto a CNBr-activated nitrocellulose membrane (0.45 μm , BAS 45 Schneider and Schuell, Germany) at 0.8 mA/cm^2 for 30 min by semi-dry blotting (Demeulemester et al., 1987; Kyhse-Andersen, 1984). The blots were washed in TBS (0.1 M Tris–HCl, pH 7.4, 0.1 M NaCl, 2.5 mM MgCl_2) containing 0.05% Tween for 30 min, dried and stored between filter papers until use. Prior to incubation of the blots with mouse serum, the membranes were incubated for 30 min with blocking buffer, TBS containing 0.05% Tween and 1% BSA for IgE antibody detection or

TBS containing 0.05% Tween and 2% nonfat dry milk (Carnation, Glendale, CA) for detection of IgG. Murine antibody reactivities were detected by incubation of blots with diluted mouse sera for 2 h, washed three times with TBS buffer, followed by incubation with alkaline phosphatase conjugated Rat anti-mouse IgE (Southern Biotechnology Associates) diluted 1:1000 for IgE antibody detection or alkaline phosphatase conjugated Goat Anti-Mouse IgG (Jackson Immune Research Laboratories, West Grove, PA) diluted 1:100,000 for IgG antibody detection. The serum and detection antibodies were diluted with appropriate blocking buffer for each assay.

To visualize the IgE-binding proteins, the blot was washed three times for 10 min in TBS-T, and washed once for 5 min in 37 °C warm TBS-AP (0.1 M Tris–HCl, 0.1 M NaCl, 5 mM MgCl_2 , pH 9.5). The antibody binding was visualized at 37 °C using the substrate/chromogen mixture for alkaline phosphatase (Leary et al., 1983) containing 450 μM 5-bromo-4-chloro-indolyl-phosphate disodium salt (BCIP; Sigma) and 400 μM nitroblue tetrazolium chloride (NBT; ICM Biomedical) dissolved in TBS-AP. The reaction was stopped with deionized water and the blots were dried.

3. Results

IgG and IgE antibody levels in shrimp (Table 1) or peanut (Table 2) immunized mice prior to and fol-

Table 2

Peanut-specific IgE antibody levels in unabsorbed and IgG-depleted (absorbed) mouse sera

Mouse strain	Unabsorbed		Absorbed		IgG removal (%)	Change of IgE detection
	IgG [$\mu\text{g/ml}$]	IgE [$\mu\text{g/ml}$]	IgG [$\mu\text{g/ml}$]	IgE [$\mu\text{g/ml}$]		
C ₃ H/HeJ	2050.0	1.5	31.1	4.6	98.4	+206.7% = 3.1 $\times$
CBA/J	344.0	2.5	35.3	8.6	89.7	+244.0% = 3.4 $\times$
Balb/c	32.0	0.4	1.9	1.6	94.0	+300.0% = 4.0 $\times$
C57Bl/6J	12.0	0.5	1.5	1.3	87.5	+160.0% = 2.6 $\times$

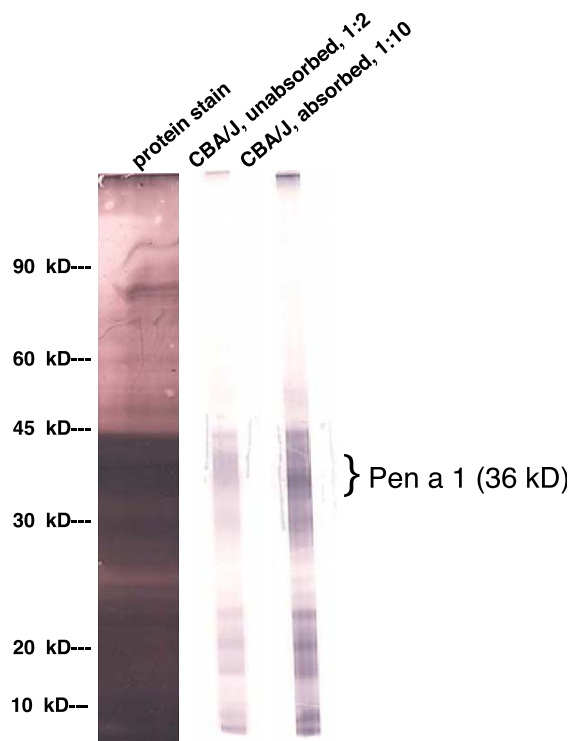


Fig. 1. Detection of IgE antibody reactivity to shrimp allergens (including Pen a 1) with sera from shrimp immunized mice absorbed with protein G (IgG depleted 94.1%) by Western blot.

lowing absorption with protein G to remove mouse IgG are summarized. Shrimp-specific IgE antibody levels ranged from 0.3 to 4.4 $\mu\text{g/ml}$; the highest IgE antibody levels to shrimp were detected in $\text{C}_3\text{H/HeJ}$ and CBA/J mice. Following absorption of these sera with protein G, 91.5% to 97% of the shrimp-specific IgG antibody was removed. However, in these same absorbed sera, there was a substantial increase in sensitivity of detection of IgE antibody levels. These levels ranged from 0.8 to 18.5 $\mu\text{g/ml}$ and represented increases in detection for some cases in excess of 300% compared to unabsorbed sera. Similar results were observed when sera from peanut-immunized mice were tested for peanut-specific IgG and IgE antibodies. Initial IgE levels ranged from 0.5 to 1.5 $\mu\text{g/ml}$; similar to shrimp-immunized mice, CBA/J and $\text{C}_3\text{H/HeJ}$ mice had the highest levels of IgE antibodies. The protein G-absorbed mouse sera, while demonstrating a substantial decrease in peanut-specific IgG (ranging from 87.5% to 98.4%) demonstrated a

significant IgE antibody increase (1.3 to 8.6 $\mu\text{g/ml}$) for all strains of mice tested. This reflected a 166.0 to 300.0% increase in detectable peanut-specific IgE antibody levels. The effect of IgG depletion on enhancement of IgE antibody measurements was consistent regardless of mouse strain, amount of initial IgE levels, or allergen (peanut versus shrimp) tested.

The effect of IgG removal on detection of murine IgE antibody levels are shown by immunoblot analysis (Figs. 1 and 2). Absorption of sera from shrimp-immunized CBA/J mice resulted in an increased reactivity to shrimp proteins. The reactivity of the absorbed serum is more intense even though it was diluted 1:10, whereas the unabsorbed serum was only diluted 1:2. Similar results obtained with sera (diluted 1:10) from peanut-immunized mice (Fig. 2); both absorbed sera from CBA/J and $\text{C}_3\text{H/HeJ}$ mice demonstrated enhanced reactivity to peanut allergens. This enhanced reactivity does not appear to be specific for any one component but reflects a general increase in detected IgE antibody reactivity to components identified within either the crude shrimp or peanut extracts assayed. It is of interest to note that there is significant

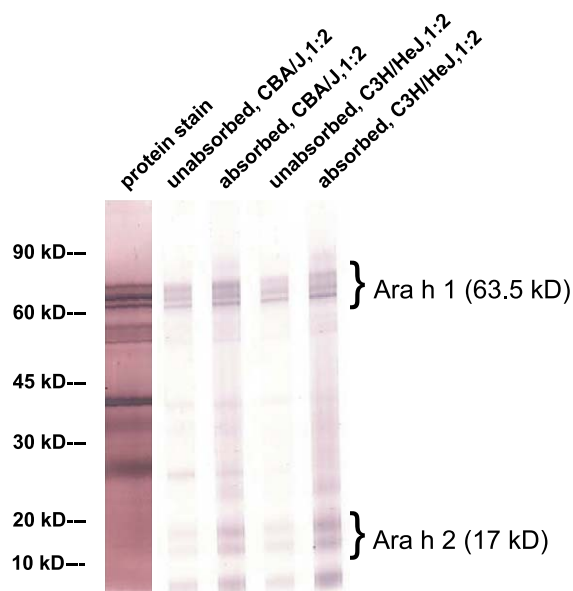


Fig. 2. IgE antibody reactivity to peanut allergens (including Ara h 1 and Ara h 2) with sera from peanut immunized mice absorbed with protein G (IgG depleted) as detected by Western blot. For CBA/J antisera, 89.7% of IgG was removed; for $\text{C}_3\text{H/HeJ}$ antisera, 98.4% IgG was removed.

reactivity of mice to the major shrimp allergen, Pen a 1, as well as the major peanut allergens, Ara h 1 and Ara h 2.

4. Discussion

Due to the perceived increase in the development of IgE-mediated allergic diseases in populations of western countries, as well as concerns within these populations of exposure to novel proteins that some consider potential allergens, there is increased interest with regard to IgE antibody production and allergy and the ability to identify allergenicity in animal models. IgE antibody production in mice has been well established for over three decades and the mouse has been an attractive model for investigation of immunologically related diseases. The development of a sensitive, reproducible, easy-to-perform in vitro assay for mouse IgE will facilitate such studies. Our findings indicate that IgG antibodies inhibit IgE antibody binding to allergen in both enzyme-linked immunosorbent assay (ELISA) as well as immunoblot analysis since removal of IgG results in enhanced IgE antibody binding. This increase in IgE antibody reactivity was shown in four different strains of mice immunized with two different allergens over a variety of IgE levels. The underestimation of IgE antibody in unadsorbed serum is due to the competition between IgE and IgG antibodies for the limited amount of antigen bound to the ELISA wells; thus murine IgG antibody reacts to the same or similar epitopes inhibiting the binding of IgE antibody.

Competitive binding between IgG and IgE in clinical assays is not a problem since these assays use antigen attached to solid phase such as cellulose sponge or particles with antigen excess binding capacity for IgE antibodies. Although removal of IgG antibodies by protein G absorption is a relatively simple procedure, it increases assay sensitivity in both ELISA and immunoblot analysis for measurement of allergen-specific mouse IgE. Although a dose response was not performed in our study, the absorptions of IgG were monitored in order to identify the level of reduction of IgG that consistently yields better IgE detection. It has been our experience that removal of more than 90% of IgG antibodies insured that IgE levels that were not detectable in unabsorbed sera

could be reliably detected. The ratio of sera to immobilized protein G varied in our studies, according to the amount of sera used. For example, for diluted sera, an equal ratio by volume of absorbent to sera was sufficient in one or two absorptions. However, with undiluted sera, absorption had to be repeated 3–6 times in order to achieve sufficient removal of IgG. It is important to emphasize that absorbed sera must be monitored for residual IgG antibody levels in order to demonstrate depletion prior to assaying IgE antibody reactivities.

In conclusion, these studies demonstrated that removal of allergen-specific IgG antibodies results in increased detection of IgE antibody levels. Thus, this simple procedure enhances the attractiveness of the mouse model of IgE antibody production for the study of allergic reactions. Clearly, if the production of IgE antibodies in mice to known allergens of man corresponds to human sensitization, animal models may be used as a first step in the evaluation of new or novel proteins to which no prior human exposure has occurred in regard to assessing their allergic potential in man. In addition, such models may also be of value for assessing the safety of new vaccines to treat allergic diseases in man.

Acknowledgements

The authors wish to thank Philip Morris USA, The Dow Foundation and Syngenta for support of this study and Pat Constant for her assistance in the preparation of the manuscript.

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