

A Method for Measuring Specific IgE in Sera by Direct ELISA without Interference by IgG Competition or IgG Autoantibodies to IgE

Yukio Kadooka^a Tadashi Idota^a Hiromi Gunji^a Masaharu Shimatani^a
Hiroshi Kawakami^a Shun-ichi Dosako^a Tomoji Samori^b

^aNutritional Science Laboratory, Snow Brand Milk Products Co. Ltd., Kawagoe, Saitama, and

^bSamori Children's Clinic, Osaka, Japan

Key Words

Specific IgE · ELISA · RAST · Protein G · Autoantibodies

Abstract

Background: In measuring specific IgE levels in sera by direct ELISA, competition with coexisting IgG often impedes an exact IgE determination; additionally, IgG autoantibodies to IgE (IgG-IgE) in sera affect the assay. In this paper, we attempt to determine accurate specific IgE levels by selective removal of IgG with a protein G-immobilized gel (PG) and by acid treatment of the PG to compensate for the unintended removal of IgE, probably due to the PG binding IgG-IgE. **Methods:** IgG in sera was removed using PG at pH 7.0. Then, the PG was treated with citrate buffer at pH 3.0 for 5 min to liberate IgE from IgG-IgE complexes, after IgG-binding sites on the PG were saturated with bovine IgG, since PG came to bind IgE at acidic pHs. IgE levels were then measured by ELISA. **Results:** The PG treatment of sera removed the effect of inhibitory competition by coexisting IgG, especially at higher concentrations of sera, to improve specific IgE detection by direct ELISA. However, PG treatment alone sometimes reduced IgE levels (39% of sera tested), even though PG does not bind IgE at pH 7.0, which indicated the presence of IgG-IgE complexes. The

reduction in IgE returned almost to their original levels in the sera by acid treatment of the PG. By combining the PG treatment with acid treatment, specific IgE measurement in sera was improved significantly ($p < 0.01$, Wilcoxon signed rank test). **Conclusion:** Measurement of specific IgE in sera by direct ELISA was improved by using the PG and acid treatment technique.

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Introduction

Since the discovery that immunoglobulin E (IgE) is the key antibody in allergic reactions [1–3], the clinical importance of determining allergen-specific IgE has been widely recognized. In current, routine clinical tests to determine allergen-specific IgE levels, the CAP-RAST system from Pharmacia is widely used, and the measurement obtained with the CAP-RAST system is expressed in RAST units (Ua) [4, 5]. To determine allergen-specific IgE levels with the CAP-RAST system requires an 'allergen CAP', a commercially provided cellulose polymer densely conjugated with various allergens designed for the system, as well as particular equipment. Thus, in analyzing specific IgE, it is more convenient to use a microplate for enzyme-linked immunosorbent assays (ELISAs).

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Correspondence to: Dr. Yukio Kadooka
Nutritional Science Laboratory
Snow Brand Milk Products Co. Ltd.
1-1-2 Minamidai Kawagoe, Saitama 350-1165 (Japan)
Tel. +81 492 42 8111, Fax +81 492 42 8696

However, as we describe here, reduced values of specific IgE were sometimes observed when sera were directly applied to ELISA microplates with immobilized allergens (direct ELISA). One reason for the reduced values is that ELISA microplates cannot immobilize allergens as densely as the allergen CAP. As a consequence, those allergens immobilized on ELISA microplates are bound largely by specific IgG that exists in sera at approximately 10,000 times the concentration of IgE. Thus, specific IgE cannot bind sufficiently to the immobilized allergens because of this phenomenon of competition [6–8]. In the CAP-RAST system, the allergen CAP provides densely immobilized allergens, to keep IgE-binding areas even in the presence of high concentrations of specific IgG [5].

One of the methods in ELISA for avoiding such a competitive interference by specific IgG is the capture ELISA technique. With this method, anti-IgE antibodies immobilized on ELISA microplates capture IgE in sera, before labeled allergens are added to detect allergen-specific IgE among the captured IgE antibodies [9–12]. However, the immobilized anti-IgE antibodies capture all IgE antibodies regardless of their specificity; as a result, another competition for immobilized anti-IgE antibodies can occur between specific IgE and nonspecific IgE, which can again lead to inaccurate determination of specific IgE. Another method involves utilizing ammonium sulfate precipitation to remove IgG in sera [13]. With this method, however, the conditions for the ammonium sulfate precipitation are strict, and may not be manageable, especially with a small amount of sera.

Thus, although there are several methods available, none is ideal; we report here a simple method to determine specific IgE levels in sera by direct ELISA with microplates. We describe the determining of specific IgE levels after selective removal of IgG in sera with protein G, a bacterial cell wall protein isolated from group G streptococci that has great affinity and specificity for most mammalian IgG [14–17]. Recently, a protein G-immobilized gel (PG), a solid support densely packed with immobilized protein G, has become commercially available, permitting an easier IgG removal operation. However, this IgG removal unexpectedly reduced detection of IgE. As this phenomenon strongly suggested the presence of IgG anti-IgE autoantibodies in sera, probably in the form of IgG-IgE complexes, we also developed a procedure to liberate IgE from such IgG-IgE complexes bound to the PG.

By combining the PG treatment and IgE liberation procedure, we determined accurate specific IgE levels in sera by direct ELISA with reduced competitive interference of IgG or the unintended removal of IgE.

Materials and Methods

Sera from Allergic Patients

Sera from patients allergic to cow's milk were obtained from patients attending the Samori Children's Clinic, Osaka, Japan. Of the sera, 38 samples were used in experiments. These patients were already diagnosed by the clinical RAST test for allergies to cow's milk; RAST values ranged from 1.01 to 85.67 Ua/ml. These values are used in this paper as cow's milk Ua.

PG Treatment of Sera

Sera (40 μ l) and 40 μ l of PG, diluted 1:2 with PBS, were mixed and incubated with shaking for 1 h at room temperature. After removing PG by centrifugation (15,000 g for 1 min), 50 μ l of supernatant was collected.

Acid Treatment of PG

To the PG suspension (30 μ l) remaining after the PG treatment of sera described above 10 μ l of 40 mg/ml bovine IgG (Sigma, USA; reagent grade) was added. This mix was incubated for 1 h at room temperature, to saturate IgG-binding sites of the PG. Then, 40 μ l of 0.1 M citrate (pH 3.0) was added to the suspension and incubated with shaking for 5 min at room temperature. The mix was then centrifuged (1 min at 15,000 g) and 50 μ l of supernatant was immediately collected and was combined with another 50 μ l of supernatant collected with the previous procedure. The combined supernatant (100 μ l) was neutralized with 50 μ l of 0.2 M sodium phosphate (dibasic)-sodium hydroxide buffer (pH 11.5).

Determination of IgE by ELISA

Wells of 96-well ELISA microplate (MaxiSorp, Nunc, Denmark) were coated overnight at 4 °C with 50 μ l of an antigen or antibody solution. For the measurement of specific IgE, skim milk (Snow Brand Milk Products, Japan) was used, and for total IgE, anti-human IgE (Seikagaku Corp., No. 260727) was used. These were dissolved in 0.1 M sodium phosphate buffer (pH 9.0) at a concentration of 10 μ g/ml. After washing the wells 3 times with PBS containing 0.05% Tween 20 (PBS-T), the remaining binding sites in the wells were blocked with 0.2 ml of 1% bovine serum albumin (Sigma) dissolved in PBS-T (PBS-TB) for 1 h at room temperature. After washing, sera or supernatants (50 μ l) were added to the wells and incubated for 2 h at room temperature. Then, after washing, 50 μ l of peroxidase-labeled anti-human IgE (Seikagaku Corp.; No. 280914) diluted in PBS-TB at 1 μ g/ml was added to the wells and incubated for 1 h at room temperature. The wells were washed and followed by the addition of 0.1 ml of QuantaBlue™ fluorogenic peroxidase substrate (Pierce Chemical Co.). After incubation at room temperature for 60 min, fluorescence intensities were measured at an emission of 440/40 nm with an excitation of 320/30 nm, using a CytoFluor Series 4000 (PerSeptive Biosystems, USA). As a standard IgE, the human IgE reagent supplied with the CAP-RAST test kit (Pharmacia) was used and was applied to the wells with immobilized anti-human IgE. The fluorescence intensity measured was normalized to the standard IgE levels and expressed in units (Ua/ml). Results represent the mean of triplicate measurements. The standard deviation did not exceed 5% of the mean.

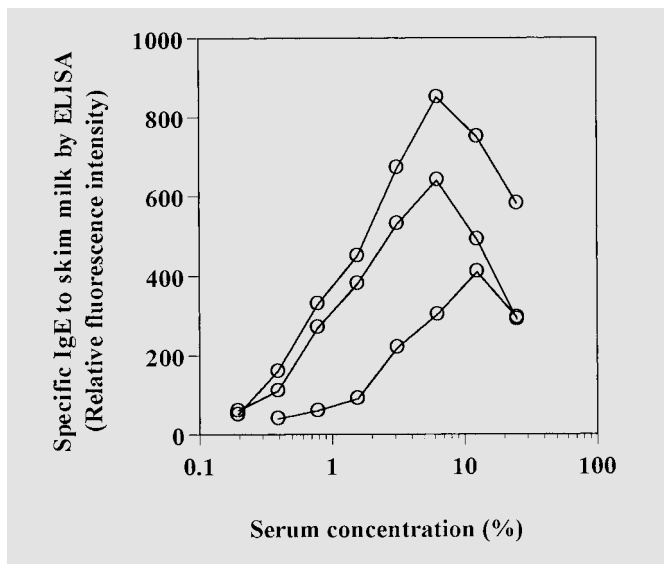


Fig. 1. Decreased specific IgE detection at higher serum concentrations by direct ELISA. Serial dilutions of sera from allergic patients who were milk RAST-positive were applied to the microplate coated with skim milk without any treatment of the sera.

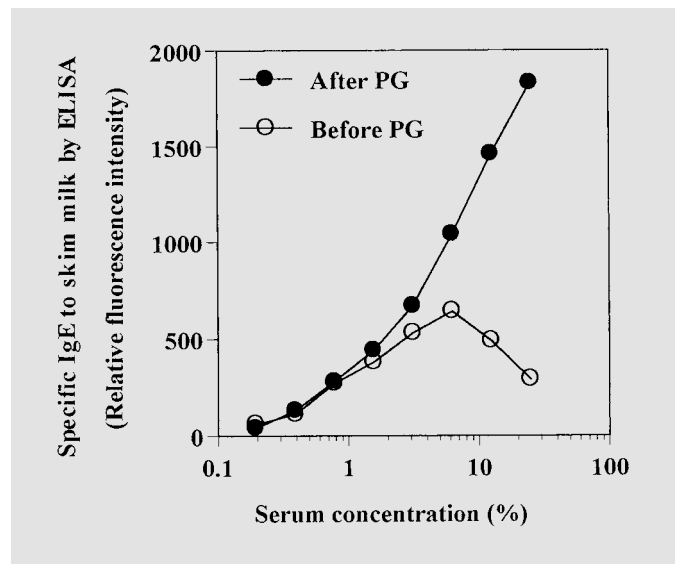


Fig. 2. Improved specific IgE detection by PG treatment of sera. A serum that showed reduced specific IgE detection at higher serum concentrations by direct ELISA was treated with PG at pH 7.0 for 1 h. After removal of the PG by centrifugation, the supernatant obtained was applied to the direct ELISA to measure specific IgE to skim milk.

Results

Elimination of Competitive Interference by IgG with the Treatment of Sera with PG

Serial dilutions of sera from patients allergic to cow's milk were directly applied to the 96-well ELISA microplate immobilized with skim milk. Figure 1 shows several typical examples of reduced specific IgE detection. At higher serum concentrations particularly, a reverse relationship between specific IgE detection and serum concentration was observed. In order to determine whether the reduced specific IgE detection was due to competition between specific IgE and specific IgG, the specific IgE was measured after the PG treatment of the sera at pH 7.0. As shown in figure 2, the reduction in specific IgE detection was overcome and correlated with serum concentrations.

Liberation of IgE from IgG-IgE Complex Bound to PG

While specific IgE detection was improved by the PG treatment (fig. 2), we observed some cases in which IgE levels were decreased by the PG treatment (as will be described later in fig. 3). Protein G is reported to bind IgG specifically, and we actually confirmed that no IgE binding to PG occurred at pH 7.0. Therefore, the unexpected decrease in IgE levels strongly suggested that the PG

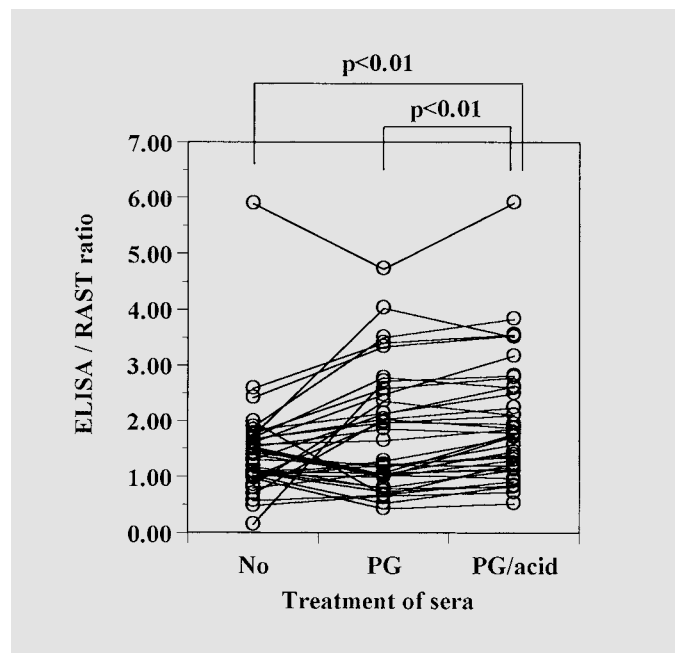


Fig. 3. Effect of PG and acid treatment of sera on skim milk-specific IgE detection by direct ELISA. Sera were subjected to no, PG or PG and acid treatment, as described in Materials and Methods. Data obtained by direct ELISA (Ua/ml) were divided by the corresponding milk RAST unit (Ua/ml) and were expressed as the ELISA/RAST ratio. Differences between treatments were compared by the Wilcoxon signed rank test.

Table 1. Optimum conditions for the recovery of IgE levels by acid treatment of PG

pH	Time of acid treatment, min		
	5	10	20
7.0	70	70	70
4.0	70	70	70
3.5	87	87	88
3.0	101	92	87

PG was reacted with pooled sera that had shown decreased IgE levels following PG treatment and was then subjected to acid treatment. Total IgE liberated in the supernatant was measured by ELISA. Values are expressed as a percentage of total IgE measured without PG treatment (= 100).

bound to IgG antibodies that were autoantibodies to IgE and formed IgG-IgE complexes in sera.

As a result, an acid treatment technique was applied to liberate IgE from IgG-IgE complexes bound to the PG. We preliminarily confirmed that PG was able to bind IgG at acidic pHs (3.0–4.0) and this binding was stable. However, at acidic pHs, the PG was apparently able to bind IgE as well as IgG (data not shown). This means that liberated IgEs can rebind to PG at acidic pHs. Thus, before the acid treatment, the IgG-binding sites on the PG were saturated with a concentrated bovine IgG (IgG blocking) in order to prevent the IgE rebinding. We confirmed that this IgG blocking was quite effective (data not shown). Then, the optimum conditions of the acid treatment (including IgG blocking hereafter) were examined. As shown in table 1, the acid treatment at pH 3.0 for 5 min was most effective at recovering the original IgE levels. Actually, as shown in table 2, several sera that showed decreased IgE levels as a result of PG treatment almost recovered their original IgE levels following the acid treatment. This result indicated that the decrease in IgE levels was chiefly due to the binding of IgG-IgE complexes to the PG.

Improved Specific IgE Detection by the PG and Acid Treatment of Sera

The effectiveness of the PG and acid treatments was examined using 38 sera whose milk RAST measurements were already known. The skim milk-specific IgE levels (Ua/ml) measured by direct ELISA were divided by the

Table 2. Recovery of total IgE levels by acid treatment of PG

Sera	Treatment	
	PG	PG and acid
A	70	101
B	89	104
C	72	109
D	87	97
E	83	96
F	103	102

PG was reacted with sera that had shown decreased total IgE levels following PG treatment and was then subjected to acid treatment at pH 3.0 for 5 min. Total IgE liberated in the supernatant was measured by ELISA. Values are expressed as a percentage of total IgE measured without PG treatment (= 100).

corresponding milk RAST unit (Ua/ml) determined in a clinical IgE test, and the results were expressed by an ‘ELISA/RAST ratio’ as shown in figure 3. The individual ELISA/RAST ratios in the no treatment group were altered by PG treatment alone – the ratio increased in 61% of sera and decreased in 39% of sera. But, as a whole, this alteration resulted in no statistical significance. On the other hand, PG and acid treatment significantly increased the ELISA/RAST ratio of the no treatment group ($p < 0.01$, Wilcoxon signed rank test) even when considered as a whole – the ratio increased in 82% of sera. In addition, the acid treatment itself also significantly increased the ELISA/RAST ratio of the PG group ($p < 0.01$).

Discussion

It has been reported that a specific IgG with the same specificity as a specific IgE in serum can interfere with an accurate measurement of specific IgE [6–8]. As a result, if specific IgE levels in sera are measured without any pre-treatment of the sera, especially when measured by direct ELISA using conventional microplates, it is possible that an accurate measurement of specific IgE levels will not be obtained, because a specific IgG – which predominates in sera – will compete for the small amount of immobilized antigen on the microplates. In the present paper, reduced specific IgE measurements were actually observed in sev-

eral sera when the sera were directly applied to the ELISA microplate coated with skim milk antigen. These decreased specific IgE measurements were rectified when the sera were treated with PG, which specifically binds to IgG. This result shows that the inhibitory competitive effect on the specific IgE measurement was chiefly due to IgG. Thus, the PG treatment of sera can readily prevent the inhibitory competition of IgG and make it possible to measure specific IgE levels in sera by direct ELISA using conventional ELISA microplates.

In some cases, however, the PG treatment of sera itself caused a decrease in the IgE measurements. We confirmed that the PG bound only to IgG and not to IgE at pH 7.0, although it came to bind IgE and IgG at acidic pHs. As the PG treatment of sera was carried out at pH 7.0, it is difficult to believe that the PG directly bound to IgE. Rather, it is more likely that the decrease in the IgE levels after PG treatment was due to the existence of IgG autoantibodies to IgE in sera, which formed IgG-IgE complexes. Presumably, the PG then bound these IgG-IgE complexes through the IgG portion, resulting in the decreased IgE levels.

Autoantibodies to IgE are known to exist [18–26] and are believed to have various physiological functions, including modulation of IgE synthesis and allergic reactions, and participation in protection against parasites [23, 27–31]. They have also been reported to inhibit the measurement of IgE levels in a clinical IgE test [21, 32–35]. On the other hand, as described above, the PG treatment was a necessary and effective method for preventing the inhibitory competitive reaction of IgG in the specific IgE measurement by direct ELISA. However, it sometimes caused the unexpected elimination of IgE. Thus, it was necessary to try to recover the ‘lost’ IgE, if the PG treatment was to be used. In order to recover the lost IgE,

we employed an acid treatment [21], and developed optimal conditions in combination with the IgG blocking.

In actual sera, it is natural to suppose that individual sera differ in their content of competitive IgGs or IgE-IgG complexes. PG treatment alone can prevent the inhibitory competition by IgGs; however, it causes a loss of IgE that bind to IgG autoantibodies if present. Our present data show that at least 61% of the sera with an increased ELISA/RAST ratio contained competitive IgGs, and at least 39% of the sera with a decreased ELISA/RAST ratio contained IgE-IgG complexes. However, it is not clear which factor – competitive IgGs or IgE-IgG complexes – is dominant in a serum.

Consequently, the combination of PG treatment with acid treatment can eliminate the effect of interference by both the IgG competition and IgG autoantibodies. In several cases (18%), however, PG and acid treatment of sera could not increase the ELISA/RAST ratio. This may be attributed to the diversity of affinity in IgG-IgE complexes, resulting in an insufficient dissociation of IgE from them. In addition, higher ELISA/RAST ratios were observed even in the no treatment group. Several causes can be considered including a difference in serum dilution at the time of reaction or in some properties of immobilized allergen used such as a state of denaturation of the immobilized allergens, which may be derived from a different method for immobilization and/or a different solid support. But we cannot give a clear explanation of this matter.

In summary, we have developed a method for measuring specific IgE in sera by direct ELISA using conventional microplates with reduced inhibitory influences of competitive IgG by using PG treatment and of IgG autoantibodies to IgE by acid treatment.

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