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The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses

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Abstract

The IgE-Facilitated Allergen Binding (IgE-FAB) assay represents an *in vitro* model of facilitated allergen presentation. Allergen-IgE complexes are incubated with an EBV-transformed B-cell line and complexes bound to CD23 on the surface of cells are detected by flow cytometry. Addition of serum from patients who have received allergen-specific immunotherapy has been shown previously to inhibit allergen-IgE complex binding to CD23 on B cells.

In this study, we describe the characterisation and analytical validation of the grass pollen-specific IgE-FAB assay according to guidelines from the International Conference on Harmonisation. We established the intra and inter -assay variability of IgE-FAB and have defined the detection limits of this assay. We have also demonstrated assay linearity and robustness. Using results from a randomised double-blind placebo controlled trial of grass pollen immunotherapy (n=33), we have defined the clinical sensitivity and specificity of the IgE-FAB assay using ROC curve analysis.

In conclusion, the IgE-FAB assay is reproducible, robust, sensitive and specific method suitable as a tool for monitoring inhibitory antibody function from patients receiving allergen immunotherapy.

Keywords

IgE-facilitated allergen presentation; Allergen-specific immunotherapy; Allergic rhinitis; IgG blocking antibodies; Assay validation

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1. Introduction

Specific allergen immunotherapy (IT) is an effective treatment for IgE-mediated allergic disease, and is particularly effective for severe seasonal allergic rhinitis (Bousquet *et al.*, 1998). The mechanisms that are associated with successful immunotherapy involve both T- and B-cell mediated events (Till *et al.*, 2004). Cellular changes include immune deviation favouring T helper (Th) 1 responses (Wachholz *et al.*, 2002), and the induction of regulatory cells producing interleukin (IL)-10 (Francis *et al.*, 2003). Treatment is associated also with the induction of allergen-specific serum IgG4 antibodies (Gehlhar *et al.*, 1999; Jutel *et al.*, 2003; Nouri-Aria *et al.*, 2004). IgG4 antibodies are thought to have blocking activities as they compete with IgE for binding to mast cells, basophils and other IgE receptor-expressing cells (Garcia *et al.*, 1993).

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 resulting in effective T cell activation at low concentrations of allergen (van der Heijden *et al.*, 1995). Formation of allergen-IgE complexes occurs at mucosal surfaces following aeroallergen exposure and results in a marked (10–100 fold) reduction in threshold levels of allergen required to trigger T cell responses. Interference with these IgE-dependent mechanisms by ‘blocking’ IgG antibodies may down regulate T cell responses and manifest as a reduction in allergic responses *in vivo*. Previous studies have shown that serum obtained from subjects following birch immunotherapy was able to inhibit IgE-facilitated presentation of allergen by B cells to an allergen-specific T cell clone (van Neerven *et al.*, 1999; van Neerven *et al.*, 2004). We have previously demonstrated that serum obtained from patients receiving grass pollen immunotherapy can inhibit IgE-facilitated allergen presentation to a grass-specific T lymphocyte clone (Wachholz *et al.*, 2003). Moreover, using a simplified assay where allergen-IgE complexes bound to the surface of B cells were detected by flow cytometry (IgE-FAB), we demonstrated that the vigour of proliferative responses by T cell clones reflect the binding of allergen-IgE complexes (Wachholz *et al.*, 2003). Using this assay we further established that inhibition of allergen-IgE binding occurs with the addition of serum from subjects receiving grass pollen immunotherapy (Wachholz *et al.*, 2003 and Nouri-Aria *et al.*, 2004).

We describe here a comprehensive analytical validation of the IgE-FAB assay, using grass pollen as an allergen and following the International Conference of Harmonization (ICH) guidelines for validation of analytical procedures (ICH guidelines, 1997). Using these guidelines, we have determined assay reproducibility, analytical sensitivity and specificity, assay linearity, intra- and inter-assay precision and robustness of the IgE-FAB assay. We have defined the clinical sensitivity, specificity, positive predictive value, negative predictive value and the likelihood ratio of the assay. For this purpose data from a randomised double-blind placebo controlled trial of grass pollen immunotherapy (Walker *et al.*, 2001) has been used.

2. Materials and Methods

2.1 Reagents

RPMI 1640 medium with L-glutamine and penicillin/streptomycin were purchased from Invitrogen (Paisley, UK). Foetal calf serum (FCS) was purchased from PAA Laboratories (Yeovil, UK). EBV-transformed B cell line and the *Phleum pratense* (*P. pratense*) allergen extract were a kind gift from ALK-Abelló (Hørsholm, Denmark). Indicator serum, containing a high concentration of grass pollen specific-IgE (RAST >100IU/ml), was purchased from PlasmaLabs (Washington, USA). Sodium chloride, sodium phosphate (dibasic), sodium phosphate (monobasic), bovine serum albumin and trypan blue were purchased from Sigma (Poole, UK). Phycoerythrin (PE)-labelled anti-human CD23 monoclonal antibody (100mg/L), isotype control (mouse IgG1-PE, 100mg/L), fluorescein isothiocyanate (FITC)-labelled

polyclonal anti-human IgE antibody (1.9g/L), isotype control (rabbit F('abs')2/FITC, 200mg/L), unlabelled anti-human CD23 monoclonal antibody (500mg/L) were purchased from DakoCytomation (Cambridge, UK). Fluticasone propionate and salmeterol were kind gifts from GalaxoSmithKline, Stevenage, U.K.

2.2 Maintenance of cell culture

The EBV-transformed B cell line has been previously characterised (van der Heijden et al., 1993) and was maintained in vented-cap, canted-neck cell culture flasks from Falcon/VWR (Poole, UK) at 37°C, 5% CO₂ and 95% relative humidity (RH). Growth medium consisted of RPMI 1640 supplemented with 1% (v/v) L-glutamine, 10% heat inactivated FCS and 1% (v/v) penicillin/streptomycin mixture. Cells were subcultured two to three times a week to maintain cell density of 0.5–1.5 × 10⁶ cells/ml. The viability of healthy growing EBV-transformed B cells were 98% to 100%. CD23 expression on B cells was monitored in each experiment. Cells were stained with anti-CD23-PE monoclonal antibody (using 10µl for 1 × 10⁶ EBV-B cells), or an isotype control (IgG1). Intra-assay variability (n=32) and an inter-assay variability from 10 runs of assay performance were analysed and the results were expressed as the percentage of B cells bound to IgE by FACS analysis. The overall mean ± Standard Deviation (SD) and the Coefficient of Variation (CV) were calculated.

2.3 IgE-FAB and inhibition of IgE-FAB assays

Stock indicator serum (20µl) containing high concentration of grass pollen (*P. pratense*) specific IgE (>100IU/ml), was pre-incubated with 1µg/ml allergen (5µl) at 37 °C for 1 h to form allergen-IgE complexes. To test for inhibition of facilitated allergen binding we mixed an equal volume of indicator serum (20µl) with test (post immunotherapy) serum (20µl) or an equal volume of RPMI as a control. In some experiments serum samples from non-atopic patients with elevated triglycerides, bilirubin or haemoglobin (kindly supplied by Dr Michael Kemp, Royal Brompton Hospital, London) were included in this step. 1 × 10⁵ EBV-transformed B cells (5µl) were then added to the allergen–IgE mixture and incubated for 1 h at 4°C. Cells were washed and bound complexes were detected using a polyclonal human anti-IgE FITC-labelled antibody (using 20µl of a 1:50 dilution for 45 min) by flow cytometry (FACS Calibur; BD Biosciences, Rockville, M.D.). B cells were gated using forward scatter/side scatter parameters and a positive marker was set using cells incubated with indicator serum only. Five thousand cells were analysed and all samples were measured in triplicate. When analysing results for longitudinal studies of grass pollen immunotherapy it is possible to account for IgE present in the immunotherapy 'test' sample which may increase the binding of allergen-IgE complexes to B cells above that of indicator serum alone. Therefore data from the clinical trial was expressed as the percentage relative binding to allergen-IgE complexes where binding with indicator serum alone was normalised to 100%. The following formula was used to calculate the percentage relative B cell binding:

$$\% \text{ relative allergen-IgE complex binding to B cells} = (\% \text{ IgE-FAB using indicator and immunotherapy serum} / \% \text{ IgE-FAB using indicator serum only}) \times 100$$

2.4 Assay controls

An indicator serum, with a pre-determined IgE-FAB binding of approximately 60%, was used as internal control for allergen-IgE binding. In addition, serum obtained from a grass pollen immunotherapy donor, that had previously been shown to inhibit binding by 90%, was used as internal control for the inhibition of allergen-IgE binding. Results from 20 runs (different days) of binding or inhibition of IgE-FAB were used to plot a Levey-Jenings chart and Westgard rules were applied to accept or reject a run (Westgard, 2003).

2.5 Assay dependency

IgE dependency of allergen-IgE binding to B cells was assessed in three ways. Firstly, IgE was selectively eliminated from indicator atopic sera by heating for 30 min at 56°C, a procedure that inhibits IgE, but not the function of other Ig classes (Demeulemester *et al.*, 1986). Secondly, IgE-dose dependency was confirmed using serially diluted indicator sera from an atopic donor. Thirdly, sera from non-atopic donors with IgE concentrations <0.34IU/ml were tested in the assay. CD23-dependency was measured using an unlabelled anti-human CD23 monoclonal antibody. The EBV-transformed B cells were pre-incubated for 1 h at 4°C with 0, 0.1, 1 and 10µg/ml of unlabelled antibody or 10µg/ml of a matched isotype control antibody. Cells were used in the binding assay as described previously.

2.6 Assay validation using results from a clinical trial

Results were obtained from a double-blind placebo-controlled trial of grass pollen immunotherapy. Briefly, 33 subjects with severe summer hay fever were randomised into groups receiving injections of a depot grass pollen (Aquagen, ALK Abelló) vaccine (n=16), or matched placebo injections (n=17) in a rapid up dosing cluster regime. Outcome measures including hay fever symptoms, use of medication, and measurement of non-specific bronchial responsiveness have been published elsewhere (Walker *et al.*, 2001). Sera were collected during a baseline monitoring year and two years after the start of immunotherapy/placebo injections and tested in the IgE-FAB assay; results have been published elsewhere (Nouri-Aria *et al.*, 2001). The study was performed with the approval of the local ethics committee and written informed consent was obtained from all patients.

2.8 Statistical analyses

Data in the text are shown as mean $\pm$ standard deviation (SD). Within group analyses (before/after treatment) were performed with the two-tailed Wilcoxon matched-pairs signed-rank test. P values <0.05 were considered significant. Receiver Operating Characteristic (ROC, Altman, 1993) curve was constructed.

3. Results

3.1 Assay dependency

3.1.1 Allergen dose-response—Initial experiments investigated the allergen concentration required to achieve optimal binding to B cells in the presence of high IgE-containing serum and inhibition of binding in the presence of serum from subjects treated with grass pollen immunotherapy. Fig. 1A shows that optimal B cell binding occurred between 1 and 3µg/ml of grass pollen allergen. At low allergen concentrations (0.1–1µg/ml) allergen-IgE complex binding to B cells was inhibited approximately 10-fold in the presence of immunotherapy serum compared to indicator serum alone. Optimal inhibition occurred at 1µg/ml which corresponded to $84 \pm 16\%$ reduction in B cell binding.

3.1.2 IgE and CD23 dependency—We investigated the requirement of IgE on complex binding to B cells. Heated indicator serum (containing inactivated IgE) was no longer able to generate allergen-IgE complexes that could bind to B cells (Fig. 1B). Similarly, serum from non-atopic controls was unable to facilitate binding of allergen to B cells (data not shown). We next tested allergen-IgE binding to B cells using a panel of serum from atopic donors with varying levels of allergen-specific IgE as measured by the ImmunoCAP system (Sabbah A *et al.*, 1990). The degree of allergen-IgE binding to B cells correlated to serum IgE concentrations ($r=0.727$, $n=18$, $p<0.001$; Fig. 1C). Pre-incubation of EBV-transformed B cells with anti-CD23 antibody resulted in a dose-dependant decrease of allergen-IgE binding (Fig. 1D). Incubation of cells with a matched isotype control antibody did not affect allergen-IgE binding.

3.2 Assay precision

3.2.1 Intra-assay and inter-assay variability—The intra-assay (within-assay) variability of allergen-IgE binding and inhibition of binding by immunotherapy serum were determined from 32 replicates. The mean percentage allergen-IgE binding to B cells was $60 \pm 3\%$ and the mean percentage decrease in allergen-IgE binding to B cell within assay was $88 \pm 2\%$ by the addition of immunotherapy serum (Table 1).

The inter-assay (between assays) variability of IgE-FAB and inhibition of IgE-FAB by immunotherapy serum was also determined on 10 runs carried out on different days. The mean percentage IgE-facilitated allergen binding between assays was $58 \pm 7\%$ and the mean inhibition of IgE-facilitated allergen binding between assays was $91 \pm 9\%$. The intra- and inter-assay variability data is shown in Table 1 and the total coefficient of variation was 16% and 12% for binding and inhibition of binding respectively.

3.2.2 Assay sensitivity—The mean allergen-IgE binding to B cells determined in 10 replicates, in the absence of allergen, was used to define the analytical sensitivity of the FAB assay. These values were used to derive the limit of detection (LOD) which were $2.5 \pm 1\%$ for the binding to B cells in the presence of indicator serum alone and $2.8 \pm 1\%$ for binding in the presence of indicator serum and immunotherapy serum.

3.2.3 Assay Linearity—Binding of allergen-IgE complexes occurred in an IgE-containing serum dilution-dependent manner and was no longer detectable in serum diluted 1/16. Inhibitory responses induced by the addition of post immunotherapy serum were also shown to be dilution-dependent (Fig. 2).

3.2.4 Drug product interference—In order to assess potential interference (Miller and Levinson, 1996), serum containing high concentrations of haemoglobin, triglycerides and bilirubin were added separately to the post-immunotherapy serum and FAP inhibition was measured. None of these were found to influence FAP inhibition (Table 2). We also assessed possible interference from two drugs commonly used for the treatment of allergy. Neither salmetrol nor fluticasone propionate influenced allergen-IgE complex binding to B cells or inhibition of binding by addition of immunotherapy serum.

3.3 Assay robustness

3.3.1 CD23 expression on B cell lines—The mean percentage CD23 expression on the surface of EBV-transformed B cells was measured by flow cytometry. Results show that the within assay expression was $97.2 \pm 1\%$, and that the CV was 2% (n=32). The mean percentage expression of CD23 between assays was $96.6 \pm 1\%$ and the CV was 2% (n=10).

3.3.2 The effect of freezing and thawing the high IgE-containing serum and post-immunotherapy serum—Three post-immunotherapy serum samples and the high IgE-containing indicator serum were freeze-thawed 5 or 10 times before testing in the assay. Freezing and thawing of high-IgE sera or post-immunotherapy sera did not influence binding or inhibition of binding in the assay (Fig. 3).

3.3.3 The influence of EBV-transformed B cell age on assay performance—An EBV-transfected B cell line that had been in continuous culture for up to 80 days was used to assess the effect of cell culture time in assay performance. We tested both allergen-IgE binding to B cells and inhibition of binding by post-immunotherapy sera after 7, 14, 28, 45, 60 and 80 days of culture. Results demonstrate that binding remained within $\pm 2SD$ of the mean binding and remained at a similar level for up to 60 days (data not shown). Similar results were obtained when inhibition of B cell responses (by the addition of immunotherapy sera) was measured.

3.4 Clinical Sensitivity and Specificity

Using the results of a randomised double-blind placebo-controlled trial of grass pollen immunotherapy the clinical sensitivity and specificity of the IgE-FAB assay was investigated. The clinical response to treatment (Walker *et al.*, 2001) and inhibition of IgE-FAB (Nouri-Aria *et al.*, 2004) have been previously published and are summarised in Table 3. In this evaluation of sensitivity and specificity, the combined clinical and medication scores were used to identify true positive (TP) and false positive (FP) results. Relative binding data from the IgE-FAB assay were used to generate a receiver operator characteristic (ROC) curve (Fig. 4). The ROC curve describes the relationship between the clinical specificity and sensitivity at any cut off level. The area under the curve is directly proportional to the diagnostic value of the assay. Using ROC curve analysis, the area under the curve was 0.977 and the 95% confidence interval was 0.932 to 1.022 ($P < 0.001$). The assay cut off obtained by this analysis was 68.1% relative binding of IgE-FAB (compared to indicator sera alone set at 100%), which gives the optimal combination of clinical sensitivity of 98.0 % (CI = 89.2, 99.9), and specificity of 94.1% (CI = 71.3, 99.9). The calculated positive predictive value (PPV), the negative predicted value (NPV), and the likelihood ratio were 100.0%, 96.9% and 17, respectively.

4. Discussion

The aim of this study was to characterise and validate an assay that could be utilised as a relevant test of inhibitory activity from sera obtained from patients with severe allergic rhinitis treated with allergen-specific immunotherapy. The IgE-Facilitated Allergen Binding (IgE-FAB) assay is a simple flow cytometric method that detects allergen-IgE complexes bound to low affinity FcεRII (CD23) on the surface of EBV-transformed B cells (Wachholz *et al.*, 2003).

We have confirmed that inhibition of allergen-IgE binding is optimal at low allergen concentrations (van der Heijden *et al.*, 1993 and 1995; Wachholz *et al.*, 2003). Formation of large allergen-IgE complexes, in this context, is dependent on the ratio of specific-IgE antibody to Phl p (allergen) in the system. In the presence of excess allergen, formation of large IgE-allergen complexes is inhibited because of a limited number of binding sites on IgE antibody (allergen excess). However, at low concentrations of allergen, multiple IgE antibodies can be cross-linked by binding to allergen to form large IgE-allergen complexes (Wachholz *et al.*, 2004).

We have also shown that binding of allergen-IgE complexes to B cells is IgE and CD23 dependent. By heat-denaturing IgE (Demeulemester *et al.*, 1996) within indicator sera we no longer observed allergen-IgE binding. There was a significant correlation between IgE-facilitated allergen binding and allergen-specific IgE levels as measured by RAST (van der Heijden *et al.*, 1995). Pre-treating the EBV-transformed B cells with monoclonal anti-CD23 antibody resulted in complete inhibition of allergen-IgE complexes binding to EBV-transformed B cells. Wachholz and colleagues showed only a partial inhibition of IgE-FAB using an anti-CD23 antibody (Wachholz *et al.*, 2003). This may have been attributable to the use of different clones of anti-CD23 antibody between the two studies.

The IgE-FAB assay was highly reproducible. The assay was also robust and unaffected by repeat freeze-thawing of serum samples or the age of cells in culture. Using the ROC curve analysis and a cut off 68.1% relative binding, we were able to define the clinical sensitivity and specificity, positive predictive value and negative predictive value (NPV) of this assay. The results show that the assay reliably detects inhibitory activity of blocking antibodies in the actively treated group. Importantly it does not give false positive results in patients who have not received treatment.

Although the read-out of the IgE-FAB assay does not measure the antigen presenting capacity of B cells to T cells, this assay has previously been shown to be representative of this process (Wachholz *et al.*, 2003). Therefore IgE-FAB assay can provide an alternative model of facilitated allergen presentation without using T cell culture techniques that are difficult to quality control. In terms of quality control, the acceptance criteria of the IgE-FAB assay were centred on the use of assay controls for IgE-FAB (atopic serum of known allergen binding) and inhibition of IgE-FAB (post immunotherapy serum of known inhibitory quality). These controls were run in every experiment and were used to monitor assay performance (bias and imprecision). Quality Assurance protocol was adhered to by the use of Standard Operating Procedures (SOPs) to prevent operator-related variability and Good Laboratory Practice (GLP) was followed at all times.

Patients receiving allergen-specific immunotherapy have been shown to have increased concentrations of allergen-specific IgG4 antibodies in their serum (Gehlhar *et al.*, 1999;Jutel *et al.*, 2003;Nouri-Aria *et al.*, 2004;Djurup *et al.*, 1987). However assays that detect only immunoreactive IgG4 are unlikely to predict clinical efficacy (Djurup *et al.*, 1987;Muller *et al.*, 1989;Ewan *et al.*, 1993;Bodtger *et al.* 2005). Therefore, measuring IgG4 concentration is not suitable for monitoring clinical efficacy of immunotherapy. Despite this lack of correlation, previous reports have demonstrated that fractionated IgG4 antibodies in serum from patients who received grass pollen immunotherapy were responsible for the inhibition of IgE-FAB to B cells (Nouri-Aria *et al.*, 2004;Wachholz *et al.*, 2003). This suggests a functional role of IgG4 in inhibition of IgE-FAB, which may be due to changes in their specificity and/or affinity. Another role of allergen-specific IgG4 antibodies induced by immunotherapy is their ability to block allergen-induced IgE-dependent histamine release by basophils confirming the functional blocking activity of these antibodies (Ball *et al.*, 1999;Lambin *et al.*, 1993).

These data have characterised the assay using grass pollen as an allergen. This assay could also be easily adapted for use with other common allergens, in conjunction with a suitable indicator serum containing high levels of allergen-specific IgE, to test for specific inhibitory antibodies induced by therapeutic protocols. In summary, the FAP assay is reproducible, robust, sensitive and specific and has potential to be used as a tool for monitoring inhibitory antibody responses induced by allergen-specific immunotherapy. It requires straightforward methodology and it can easily be introduced into specialist immunology laboratories investigating inhibitory antibody responses induced during allergen immunotherapy.

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Abbreviations used

IgE-FAB	IgE-facilitated allergen binding
Phl p	Phleum pratense
RAST	Radioallergosorbent test
IT	Immunotherapy

Fig 1.

Assay characterisation. A. High IgE-containing sera was incubated with various concentrations of allergen in absence (open bars) or presence (closed bars) of serum obtained after 1 year of grass pollen immunotherapy (IT). Results are shown as mean ($\pm$ SD) percentage B cells bound to allergen-IgE complexes as detected by flow cytometry. B. Atopic serum was heat-inactivated at 56°C for 30 min and compared with untreated serum in the FAB assay using 1 μg/ml of allergen. This result is representative of 3 different serum samples. C. Sera was obtained from 18 grass pollen allergic subjects and tested in the FAB assay. The graph shows the correlation between binding and allergen-specific IgE levels as determined by the ImmunoCAP system. D. EBV-transformed B cells were pre-treated with anti-CD23 blocking antibody (closed symbols) or isotype control (open symbol) for 30 mins at 4°C. Allergen-IgE binding was detected using atopic serum and 3 μg/ml of allergen. All data are shown as mean ($\pm$ SD) percentage B cells bound to allergen-IgE complexes.

Fig 2.

Assay linearity. A. Atopic serum (n=3) was diluted as indicated and incubated with 1 μ g/ml of allergen and tested for binding in the assay. Results are shown as mean ($\pm$ SD) percentage B cells bound to allergen-IgE complexes. B. Serum from subjects treated with grass pollen immunotherapy (n=3) was diluted as indicated and mixed with atopic serum, 1 μ g/ml of allergen and tested for binding in the assay. Data is shown as mean ($\pm$ SD) percentage B cells bound to allergen-IgE complexes.

Fig 3.

Assay robustness. A. Serum samples from atopic (n=3) and grass pollen immunotherapy (n=3) subjects were thawed and frozen ten times and tested in the assay. Allergen-IgE binding to B cells was measured after 5 and 10 freeze-thaw cycles. Results are shown as mean ($\pm$ SD) percentage binding.

Fig 4.

Receiver operating characteristic (ROC) curve analysis of the IgE-FAB assay. Using data obtained from a clinical trial of grass pollen immunotherapy the sensitivity is plotted against 1-specificity to show the diagnostic accuracy of the assay. The cutoff value for the inhibition of IgE-FAB assay was determined as 68.1% relative binding to indicator serum and the clinical sensitivity and specificity of this assay are 98.0% and 94.1%, respectively.

Table 1**Assay precision – analysis of intra-assay and inter-assay variability**

The intra-and inter-assay precision were measured for IgE-facilitated allergen binding using an atopic serum containing high allergen-specific IgE and reduction of IgE-facilitated allergen binding using a serum obtained post-grass pollen immunotherapy.

		% of B cells binding to allergen-IgE complexes	% inhibition in B cell binding to allergen IgE complexes
Intra-assay variability	<i>Mean (n= 32)</i>	60	88
	<i>Standard Deviation</i>	3	2
	<i>Coefficient of Variation (%)</i>	4	2
Inter-assay variability	<i>Mean (n= 10)</i>	58	91
	<i>Standard Deviation</i>	7	9
	<i>Coefficient of Variation (%)</i>	12	10

Table 2**Interference by plasma constituents and common drugs used in allergy treatment**

Atopic and post immunotherapy samples were spiked with interfering substances containing common by plasma constituents and common drugs used in allergy. The effect of these interference substances were measured in the IgE-FAB assay. The normal reference ranges for the interfering substances are shown in brackets.

Interfering substances	Concentration tested	% of B cells binding to allergen-IgE complexes	% inhibition in B cell binding to allergen IgE complexes
Salmeterol (M)	10^{-5}	47	80
	10^{-6}	48	79
	10^{-7}	45	80
Fluticasone propionate (M)	10^{-8}	45	76
	10^{-10}	45	78
	10^{-12}	47	75
	>18 (13.5–18)	49	86
Haemoglobin (g/d l)			
Bilirubin (mg/ml)	>10 (2.5–10)	53	87
Triglyceride (mM)	>2 (<2)	54	80
Control serum		55	83

Table 3**Summary of clinical results and influence of serum on allergen-IgE binding to B cells**

Data shows median (IQR) of combined symptom and medication scores and inhibition of allergen-IgE binding to B cells. Groups were compared using the Wilcoxon's matched-pairs signed-rank test. Values in bold are statistically significant.

	Pre-treatment	Post-treatment	P value*
A. Combined symptom and medication scores			
Immunotherapy (n=16)	4505 (2994,7405)	1561 (178,5262)	0.019
Placebo (n=17)	4370 (1876,7392)	4263(1299,9261)	0.980
B. Allergen-IgE binding to B cells (% relative binding to indicator serum)			
Immunotherapy (n=16)	90.56 (84.60,95.92)	25.98 (10.38,39.29)	<0.001
Placebo (n=17)	89.87 (78.22,103.72)	88.27 (82.75,94.44)	0.770