

Human Allergen-Specific IgG Subclass Antibodies Measured Using ImmunoCAP Technology

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Abstract

Background: Knowledge of human IgG subclass antibody responses to various allergens has been hampered by a lack of reliable standardized assays. The aim here was to develop quantitative immunoassays for human IgG1, IgG2, and IgG3 antibodies using ImmunoCAP[®] technology and to evaluate their application. **Methods:** Enzyme conjugates with isotype-specific monoclonal antibodies and calibrators composed of purified myeloma paraproteins were developed for each assay and used together with other standardized assay reagents for the Phadia[®] 100 instrument. The calibrators were adjusted to the international reference preparation IRP 67/86. The assays were characterized and used together with other standard ImmunoCAP assays to measure antibodies to various allergens in preliminary studies. **Results:** The new assays had limits of quantitation of 1.0 (IgG1), 4.6 (IgG2), and 0.04 mg_A/L (IgG3), and coefficients of variation of <20%. Only some minor cross-reactivity with IgG2 was observed for the specific IgG1 assay. The specific IgG2 assay showed a bias for the allotype G2m(23) and compensa-

tion factors were used to adjust the measured concentrations accordingly. Preliminary studies indicated a strong and stable IgG4 antibody response to β -lactoglobulin in healthy individuals, a high IgG1 and even higher IgG2 antibody response to house dust mite in sensitized and nonsensitized subjects, and a mixed IgG subclass response to venom allergens in allergic patients with increasing IgG4 antibody levels during venom immunotherapy. **Conclusions:** The new research assays are valuable tools for immunological studies, enabling the characterization of antibody profiles using a standardized approach, and facilitating data interpretation and the comparison of results across studies.

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Introduction

Foreign proteins drive humoral immune responses in humans. This can occur via different routes of exposure (e.g. ingestion, parenteral, inhalation, dermal), and responses, in terms of the type and quantity of antibody generated, may vary with a number of parameters including, but not limited to, the antigen, dose, and timing of exposure [1].

IgG is the most predominant isotype among the 5 human immunoglobulin classes. About 8–16 mg/mL IgG is present in human serum, and antibodies of the IgG isotype normally dominate the humoral immune response to viruses and bacterial antigens. Four subclasses of human IgG have been described, named IgG1, IgG2, IgG3, and IgG4 [2]. Although these IgG subclasses show more than 90% sequence identity with each other, the rather small differences have a dramatic influence on the biological functions of the antibodies [3].

IgG1 could be considered as the most important of the IgG subclasses since it constitutes about 60% of all IgG in human serum as shown in various studies [4]. Antibodies of the IgG1 subclass dominate in immune reactions to most protein antigens [3]. IgG1 is able to activate complement and can effectively interact with Fcγ receptors present on various effector cells. IgG2 is the second most common subclass (approx. 30%) and has been reported to dominate in the response to bacterial polysaccharide antigens [5, 6]. IgG2 antibodies are poor complement activators and are bound with very low affinity by some Fcγ receptors [3]. The IgG3 and IgG4 subclasses are less common in human serum (approx. 6 and 4% of total IgG, respectively). IgG3 antibodies show the best effector functions among the IgG antibodies, characterized by their efficient complement activation capacity and very high affinity binding to all known Fcγ receptors. Finally, IgG4 antibodies are often produced later in the antibody response, at which point IgG4 can then even be the dominating subclass [7]. IgG4 antibodies have no complement activating function and bind Fcγ receptors with low affinity. IgG4 is thought to have a regulatory role in the humoral immune response [8], and a large increase in IgG4 antibodies to allergens is commonly seen after successful allergen-specific immunotherapy [9, 10].

The natural history of IgG responses to foreign antigens encountered normally in the environment or via ingestion and subsequent development of allergy and/or intolerance is not well understood. Although the roles of antibodies of different isotypes have been studied for decades, knowledge of the different IgG subclass contributions to human immune responses has been hampered by the lack of reliable standardized subclass-specific antibody assays. A major reason has been the challenge of developing suitable subclass-specific antibody reagents. The majority of the commercially available monoclonal antibodies were developed and characterized in the 1980s and early 1990s [11, 12]. Cross-reactivity between the subclasses is a general problem among them, especially when it comes to IgG1 and IgG2 [11, 12]. A complicating

matter is that several structural differences within each subclass, presented as immunogenic allotype variations, have been described for IgG1, IgG2, and IgG3 [13]. This allotype variation may affect the binding of the subclass-specific antibodies [11, 12].

The development and application of standardized immunoassays to investigate the quality and magnitude or vigor of IgG antibody responses, including all 4 subclasses, could provide insights into the mechanisms involved in the development of immunity as well as allergies and/or intolerances. Most published studies in the field have used nonvalidated enzyme-linked immunosorbent assays with semiquantitative results in the form of absorbance units, or results based on internal calibration systems without traceability to accepted international reference preparations (IRPs).

The aim of the work described here was to develop quantitative immunoassays for human IgG1, IgG2, and IgG3 antibodies using ImmunoCAP® technology including calibrators traceable to IRP 67/86 from the World Health Organization (WHO) [14, 15]. The assays were developed for use in automated Phadia® 100 instruments and were characterized in preliminary validation studies. The new research assays were used in a pilot study together with commercially available ImmunoCAP assays for specific IgG, IgG4, and IgE to measure antibodies to β-lactoglobulin (BLG) from cow's milk, house dust mite (HDM) extract, bee or wasp venom extract, cross-reactive carbohydrate determinant (CCD), and recombinant venom allergen components in 3 sets of serum samples.

Material and Methods

Study Samples

Three sets of serum/plasma samples were used in the study. Sample set A was obtained as part of a clinical study investigating immune responses to ingested proteins and consisted of 2 samples, from each of 56 healthy adult subjects without a known milk allergy, collected 85 days apart (study conducted at Charité Research Organization, Berlin, Germany). Written informed consent was obtained from each subject. Sample sets B and C were from the internal sample collection of Thermo Fisher Scientific, Immuno-Diagnostics (Uppsala, Sweden). Sample set B was composed of 12 serum and plasma samples selected for having HDM-specific IgG antibodies. None of the blood donors were documented as being HDM allergic. Sample set C was composed of serum samples from 8 honey bee or wasp venom-allergic patients collected before and during venom-specific immunotherapy (VIT). Up to 4 samples had been collected from each patient at different time points during 1 year of VIT. Ethical approval was obtained from the relevant independent ethics committees (for sample set A from the Ethic Committee Charité, Berlin, Germany, and for sample sets B and C

from the Ethic Committees of Uppsala University and University of Gothenburg, Sweden). All samples were stored at -20°C until analysis.

Development of Subclass-Specific Antibody Assays

Subclass-specific antibody assays were developed for research use based on ImmunoCAP technology and for use in automated Phadia 100 instruments (Phadia, Uppsala, Sweden). Briefly, human IgG subclass-specific mouse monoclonal antibodies were conjugated to β -galactosidase following internal methods and diluted to appropriate concentrations in ImmunoCAP Specific IgG conjugate buffer (Phadia). The specificity of each antibody-enzyme conjugate was confirmed as described below. Calibrators at 6 concentration levels covering the measuring range were developed for each assay by dilution of purified myeloma paraproteins of IgG1, IgG2, and IgG3 isotypes, respectively. The paraproteins are internal reagents of Thermo Fisher Scientific, ImmunoDiagnostics. The given concentration of each calibrator was adjusted to the IRP 67/86 for human serum immunoglobulins A, G, and M from the WHO [14, 15]. The concentrations of the IgG subclasses in the related WHO IRP 67/97 were established by Klein et al. [15]. Heterologous calibration systems were developed following the principle of the standard ImmunoCAP assays for IgG and IgG4 antibodies. The calibrators were incubated on the commercially available calibrator ImmunoCAP, a test coupled with anti-kappa and anti-lambda light chain antibodies (ImmunoCAP IgA/IgG Calibrator ImmunoCAP). After immobilization followed by extensive washing, the calibrator immunoglobulin was bound by the subclass-specific antibody-enzyme conjugate. Commercially available ImmunoCAP tests with allergen extracts and single proteins (allergen/antigen components) can be used in the developed assays to measure the allergen/antigen-specific IgG subclass antibody concentration in samples by automatic comparison of the assay responses with the calibration curves.

New software methods for the IgG1, IgG2, and IgG3 subclass antibody assays, based on the method for ImmunoCAP Specific IgG, were developed for the Phadia 100 instrument for running the assays including calibrator curve fitting and conversion of assay responses to antibody concentrations. The sample and enzyme conjugate incubation times are 30 and 24 min, respectively. The incubations, which are performed at $+37^{\circ}\text{C}$, are terminated by extensive washing steps using ImmunoCAP Washing Solution. The formed allergen/antigen-antibody complexes are proportional to the fluorescence generated after adding Development Solution and finally Stop Solution. These solutions are generic standard ImmunoCAP assay reagents. The fluorescence is measured by the Phadia 100 instrument at the end of the assay run. Values are expressed in mg_A/L , where A represents antigen-specific antibodies.

The lower limit of quantitation (LLOQ) for each assay was defined as the concentration of the lowest calibrator having an imprecision of $<20\%$ coefficient of variation (% CV). The serum or plasma samples should be diluted 1:100 in ImmunoCAP Specific IgA/IgG Sample Diluent (assay diluent) before analysis (the dilution can be automated by the Phadia 100 instrument).

Characterization of the Subclass Assays

The isotype-specificity of the developed subclass assays was confirmed by analyzing highly purified human myeloma paraproteins of the IgG1, IgG2, IgG3, IgG4, IgA, and IgM isotypes (internal reagents) at $2\text{ }\mu\text{g}/\text{mL}$ in assay diluent using the calibrator (anti-

kappa/anti-lambda) ImmunoCAP test. The concentration corresponds to a serum level of $200\text{ mg}/\text{L}$. Furthermore, the specificity for the major allotypes of each subclass were studied using monoclonal therapeutic IgG1 antibodies with known allotype expression (G1m3 and G1m17,1) and myeloma paraproteins of the IgG2 [G2m23 and G2m(..)] and IgG3 isotypes (G3m5 and G3m21) [13].

The given concentration of each calibrator was confirmed using the commercial assay ImmunoCAP Specific IgG where the subclass calibrators were analyzed as samples using the ImmunoCAP calibrator test. The assay precision of the developed subclass antibody assays was assessed using human serum samples with IgG1, IgG2, and/or IgG3 antibodies representing low, medium, and high antibody concentrations according to the measuring ranges of the assays. The samples were analyzed in 3 replicates in 5 separate assay runs. Analysis was performed during a period of 3 days on 4 different Phadia 100 instruments. The intra- and inter-assay % CV was calculated for each sample according to Cheshier [16].

Dilution linearity of the subclass assays were assessed using serum samples with high IgG1, IgG2, and/or IgG3 antibody concentrations that were diluted in 5 steps (1:1, 1:2, 1:3, 1:5, 1:10, and 1:20) in presumed negative serum samples. Each sample dilution was then further diluted 1:100 in assay diluent according to the method and analyzed using the subclass-specific ImmunoCAP assay in 4 replicates in 1 assay run per positive sample. The negative sample backgrounds were subtracted from the results before calculation of the interdilutional precision. Samples with very high antibody concentrations were not available. Therefore, to get samples covering the upper part of the measuring ranges myeloma paraproteins were used. They were diluted in assay diluent before analysis in the assays using ImmunoCAP calibrator tests. The interdilutional % CV was determined from the obtained mean concentrations multiplied with the corresponding dilution factor.

Recovery (matrix study) was assessed using 3 IgG subclass antibody-positive serum samples diluted 1:6 in 3 individual presumed negative serum samples. Each sample preparation, including the negative and positive samples, was analyzed using the subclass-specific ImmunoCAP assay in 4 replicates in 1 assay run. The antibody recovery was calculated for each sample after correction for negative sample backgrounds.

Sample Analysis

The frozen samples were thawed and gently mixed before analysis using the ImmunoCAP assays on the Phadia 100 instrument. The assays for specific IgG, IgG4, and IgE are commercially available with an LLOQ of 2 and $0.07\text{ mg}_A/\text{L}$, and $0.1\text{ kU}_A/\text{L}$ ($=0.24\text{ ng}/\text{mL}$), respectively. The assays for IgG1, IgG2, and IgG3 antibodies are research assays described in this report with an LLOQ of 1, 4.6, and $0.04\text{ mg}_A/\text{L}$, respectively. Results below the LLOQ were assigned a fixed value for statistical calculations. All of the ImmunoCAP allergen tests used are commercially available.

The G2m23 allotype expression (homo- or heterozygosity) were determined for all samples using a previously described double-diffusion assay [17]. Due to a G2m23 allotype bias of the specific IgG2 assay, the obtained IgG2 antibody results were adjusted using the multiplication factors 0.17 and 0.29 for G2m23 homozygotes and heterozygotes, respectively.

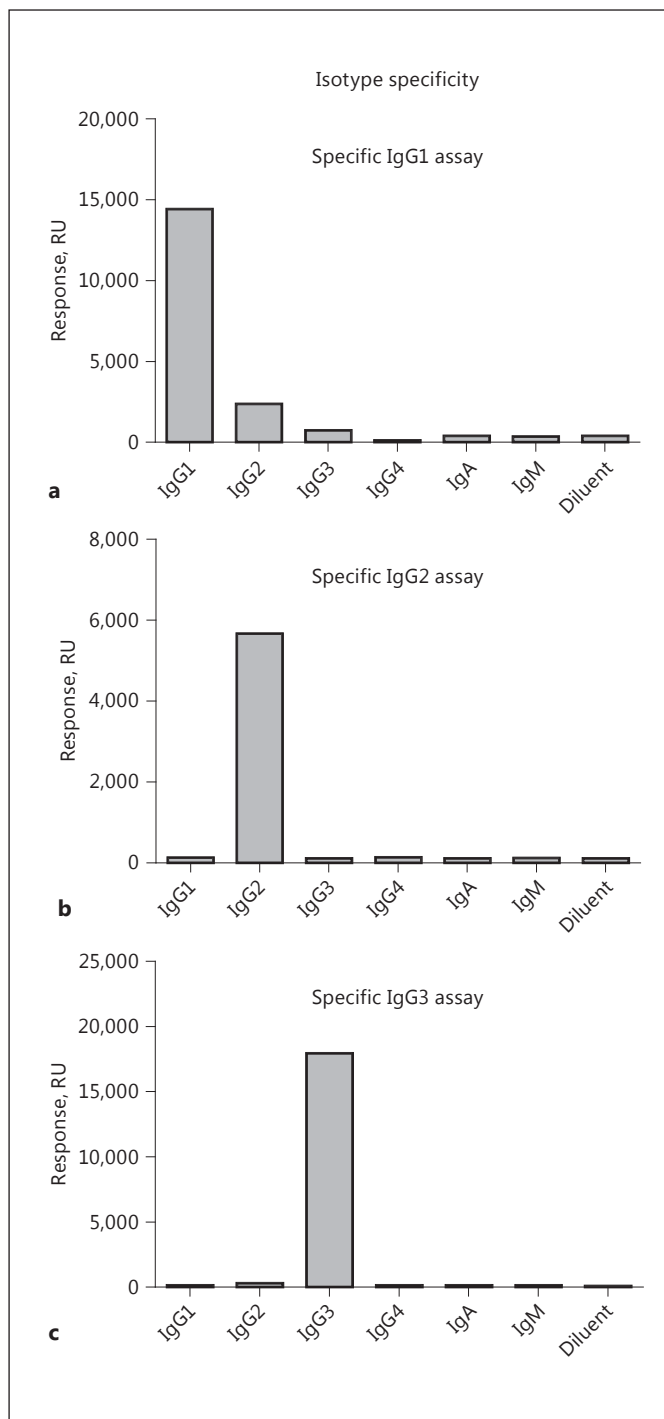


Fig. 1. Isotype specificity of the ImmunoCAP research assays for quantitative measurement of IgG1 antibodies (a), IgG2 antibodies (b), and IgG3 antibodies (c) in human serum or plasma samples. The specificity was evaluated by analyzing purified myeloma paraproteins (2 µg/mL) of various isotypes diluted in assay diluent.

Statistics

Data were analyzed using GraphPad Prism (version 5.04, GraphPad Software, La Jolla, CA, USA). The Wilcoxon matched-pairs signed-rank test was used for paired sample comparisons (within group). The Mann-Whitney *U* test (2-tailed) was used for pairwise comparisons of continuous parameters. The Spearman rank order correlation coefficient (r_s) was used to measure the strength of the relationships between antibody levels. Only values within the measuring ranges of the assays were used in the correlation evaluation. *p* values <0.05 were considered significant.

Results

Development and Characterization of the Specific IgG1, IgG2, and IgG3 ImmunoCAP Assays

Assays for IgG1, IgG2, and IgG3 antibodies were developed using the ImmunoCAP technology for the Phadia 100 instrument including automated conversion of assay responses to antibody concentrations using calibrator curves (online suppl. Fig. S1). The isotype specificity of the assays is shown in Figure 1. Some minor cross-reactivity with IgG2 was observed for the specific IgG1 assay. No other apparent cross-reactivity was observed for the subclass-specific antibody assays.

The antibody-enzyme conjugates of the specific IgG1 and IgG3 assays bound equally well to the 2 major allotype forms of IgG1 (G1m3 and G1m17,1) and IgG3 (G3m5 and G3m21), respectively (data not shown). The antibody-enzyme conjugate of the specific IgG2 assay, however, bound more efficiently to the G2m23 allotype than to the nonallotype form of IgG2 [G2m(..)] (data not shown). The calibrator IgG2 paraprotein of the assay belonged to the nonallotype G2m(..) variant. To compensate for the difference in antibody-enzyme conjugate binding, results from the specific IgG2 assay obtained for subjects homozygous for the G2m23 allotype had to be adjusted using the multiplication factor 0.17. Accordingly, results for heterozygous individuals were adjusted using the factor 0.29, assuming that both IgG2 variants were synthesized at equal levels. The multiplication factors were obtained by comparing results from the specific IgG2 assay and the specific pan-IgG assay (ImmunoCAP Specific IgG) using samples consisting of purified myeloma IgG2 paraproteins of both allotype variants. ImmunoCAP Specific IgG showed no allotype bias in the present study (data not shown).

The LLOQ was established to 1 mg_A/L for the specific IgG1 assay, 4.6 mg_A/L for the specific IgG2 assay, and 0.04 mg_A/L for the specific IgG3 assay based on an imprecision requirement of <20% CV (Table 1; online suppl. Fig. S1;

Table 1. Measuring range and characteristics of the ImmunoCAP research assays for the measurement of IgG1, IgG2, and IgG3 antibodies

	Research ImmunoCAP assay		
	specific IgG1	specific IgG2	specific IgG3
Measuring range, mg _A /L	1.0–400	4.6–100 ^a	0.04–21
Characteristics			
Intraassay precision, % CV	6.3 (2.0–10.5)	3.0 (1.2–4.4)	4.2 (3.1–6.2)
Interassay precision, % CV	8.8 (0.3–17)	9.4 (2.4–19.3)	5.0 (3.4–5.9)
Interdilutional precision, % CV	13.8 (6.2–20.8)	12.3 (10.8–13.8)	8.3 (5.1–10.8)
Recovery (matrix study), %	77 (67–88)	99 (72–118)	99 (83–121)

Values are presented as the range or mean (range).

^a The obtained values in the specific IgG2 assay should be adjusted according to the IgG2 allotype expression [multiplication factors: G2m(..)/G2m(..), 1; G2m23/G2m23, 0.17; G2m23/G2m(..), 0.29].

see www.karger.com/doi/10.1159/000455098 for all on-line suppl. material).

The given concentrations of the calibrators were verified using the commercial assay ImmunoCAP Specific IgG. The mean ratios of the obtained/expected concentrations of the calibrators analyzed as samples in the ImmunoCAP Specific IgG assay were 1.00 for the IgG1 calibrators, 1.03 for the IgG2 calibrators, and 0.81 for the IgG3 calibrators, i.e. all calibrators were <20% from expected values.

The intra- and interassay precision of the 3 subclass assays was <20% CV for the low, medium, and high samples. The mean intra- and interassay precisions for each assay are given in Table 1.

The dilution linearity of the assays, defined as the mean interdilutional precision of 3 sample dilution series, ranged from 8.3 to 13.8% CV, and the mean matrix recovery ranged from 77 to 99% (Table 1). A lower matrix recovery was observed for the specific IgG1 assay than for the other 2 assays, partly due to the negative sample background correction. Without background correction the mean recovery was 86% (range 68–123%).

IgG subclass Antibodies to BLG

Sample set A was composed of sera from 56 healthy adults with no reported milk allergy (Fig. 2a). The median concentration of total IgG antibodies to BLG was 2.2 mg_A/L (range <2–31.1) and only 31 (55%) of the samples were positive in the specific IgG assay (>2 mg_A/L). The IgG subclass antibody profile was characterized by relatively high levels of IgG4 antibodies. However, the inter-individual variation was large. Forty-one (73%) of the subjects were positive in the BLG-specific IgG4 assay (median concentration 0.53 mg_A/L, range <0.07–28.3).

Forty-two (75%) of the subjects were also positive for IgG3 antibodies to BLG, but at lower levels than the IgG4 antibodies (median 0.06 mg_A/L, range <0.04–0.54, $p < 0.0001$). Hardly any had measurable IgG1 antibodies (assay cut-off 1 mg_A/L), and none had IgG2 antibodies (assay cut-off 4.6 mg_A/L). The serum levels of specific IgG3 and IgG4 corresponded to 31% (range 5.0–102%) of the pan IgG antibody levels measured. Only 2 of the subjects had measurable IgE antibodies to BLG and at very low concentrations (0.13 and 0.34 kU_A/L). These 2 subjects had very high specific IgG4 levels (Fig. 2a). The individual antibody concentrations were similar when analyzing samples collected 85 days after the initial sample collection, a period over which milk was known to be regularly ingested by all, as shown for IgG3 and IgG4 (online suppl. Fig. S2).

IgG Subclass Antibodies to HDM

Subset B was composed of 12 sera from subjects known to have IgG antibodies to HDM (Fig. 2b). They all had very high concentrations of total IgG antibodies to HDM (median 24.4 mg_A/L, range 9.9–42.2). Six of them also expressed IgE antibodies to HDM (median 0.22 kU_A/L, range 0.10–3.72). No allergy to HDM was reported, but the clinical information about the blood donors was incomplete. The sensitized subjects had higher median antibody levels to HDM, although the difference was only significant for pan IgG ($p < 0.05$). The subclass profile of the IgG antibodies was characterized by high levels of IgG2 antibodies (median 13.2 mg_A/L, range 5.8–37.4) and slightly lower levels of IgG1 antibodies (median 4.9 mg_A/L, range 1.7–14.2, $p < 0.01$). All patients were also positive for HDM-specific IgG3. Even if the IgG3 antibody levels (median 0.29 mg_A/L, range 0.15–0.95) were much lower than the IgG1 and IgG2 antibody levels, they

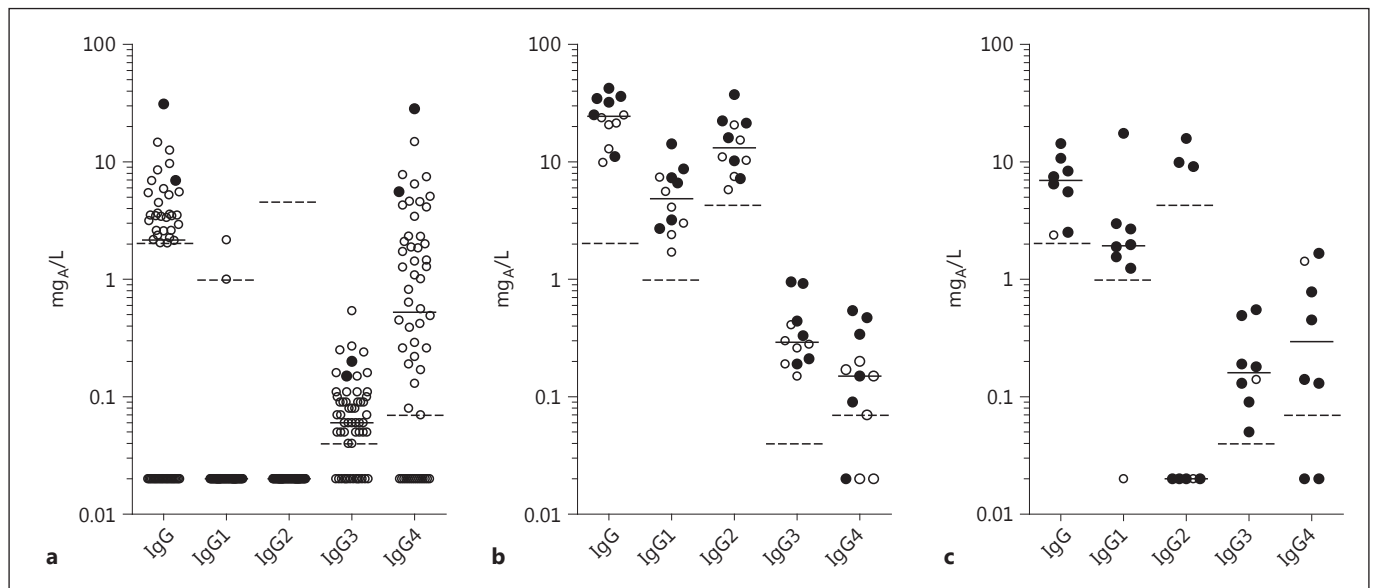


Fig. 2. Pan IgG and IgG subclass antibody concentrations to BLG in healthy subjects ($n = 56$; **a**), HDM in subjects without a known mite allergy and selected for having high specific IgG ($n = 12$; **b**), and bee or wasp venom in allergic subjects selected for venom immunotherapy (samples collected before the start of treatment,

$n = 8$; **c**). Closed and open symbols represent subjects with (>0.10 kU_A/L) and without measurable IgE antibodies to the allergen, respectively. The solid line represents the median concentration. The broken line represents the LLOQ of each assay. All results below the LLOQ were given the value of 0.02 mg_A/L.

were generally higher than the IgG4 antibody levels (0.15 mg_A/L, <0.07 –0.54 mg_A/L, $p < 0.05$). The sum of the specific IgG1, 2, 3, and 4 measurements corresponded to 86% (range 59–119%) of the measured HDM-specific pan IgG concentration.

IgG Subclass Antibodies to Bee and Wasp Venom before and during VIT

Subset C included serum samples from 4 honey bee and 4 wasp venom-allergic patients selected for VIT treatment (Fig. 2c). At the start of treatment all but 1 of the patients had IgE antibodies (>0.1 kU_A/L) to the culprit venom (bee or wasp venom extract), and all had venom-specific IgG antibodies (median 7.0 mg_A/L, range 2.4–14.3). Seven of them also had measurable IgG1 antibodies, but generally at lower concentrations (median 1.9 mg_A/L, range <1 –17.4, $p < 0.05$). Three were positive in the IgG2 antibody assay (9.1, 9.9, and 15.8 mg_A/L). The IgG3 antibodies (median 0.16 mg_A/L, range 0.05–0.55) and IgG4 antibodies (median 0.30 mg_A/L, range <0.07 –1.7) composed a minor part of the IgG response to the venom extracts.

The antibody response to bee or wasp venom during VIT was followed for up to 1 year of treatment (Table 2). It was characterized by a temporary increase in venom-specific IgE antibodies in some patients (not significant

at group level) and a gradual increase in venom-specific IgG. The latter response was dominated by IgG4 antibodies. A nonsignificant increase in IgG1 antibodies was also indicated in some patients (online suppl. Fig. S3). The patient with the largest increase in specific IgG (to bee venom) also had a major increase in venom-specific IgG1 antibodies and a smaller increase in venom-specific IgG3.

The sera from the bee and wasp venom-allergic patients were also analyzed for antibodies to CCD-free recombinant Api m 1 (from bee venom) and Ves v 5 (from wasp venom) and to CCD (Table 2). The serum IgG antibody levels to Api m 1 and Ves v 5 highly correlated with the IgG antibody levels to bee and wasp venom extract, respectively ($r_s = 0.9562$, $p < 0.0001$). Similar correlations in antibody levels to the major venom allergen components and the corresponding venom extracts were seen for all 4 IgG subclasses ($r_s = 0.8461$ –0.9695, $p < 0.0001$). The quantitative kinetic data indicated that most of the IgG antibody response during the VIT, including specific IgG4, was directed against the 2 major bee and wasp venom allergens (Table 2). A minor IgG antibody response was observed to CCD in a few patients that was mostly related to IgG3 and IgG4. The IgG response to CCD did not increase during VIT. No correlation was observed between the antibody levels to CCD and venom extract, ex-

Table 2. Antibody response (median with range) during VIT in 8 bee or wasp venom-allergic patients (sample set C)

	Specific IgG, mg _A /L	Specific IgG1, mg _A /L	Specific IgG2, mg _A /L	Specific IgG3, mg _A /L	Specific IgG4, mg _A /L	Specific IgE, kU _A /L
Venom extract						
Before VIT	7.0 (2.4 to 14.3)	1.9 (<1 to 17.4)	<4.6 (<4.6 to 15.8)	0.16 (0.05 to 0.55)	0.30 (<0.07 to 1.7)	5.2 (<0.1 to 45.4)
1 month of VIT	9.0 (<2 to 14.1)	2.3 (1.2 to 12.8)	<4.6 (<4.6 to 15.4)	0.15 (0.05 to 0.51)	0.44 (<0.07 to 2.3)	5.0 (<0.1 to 58.1)
2 months of VIT	16.1 (3.4 to 28.3)*	4.0 (<1 to 15.6)	<4.6 (<4.6 to 14.0)	0.24 (0.05 to 0.53)	2.7 (0.14 to 9.0)*	3.9 (0.16 to 69.6)
1 year of VIT ^a	19.4 (8.4 to 45.8)**	3.8 (2.0 to 16.4)	<4.6 (<4.6 to 13.6)	0.16 (0.08 to 0.39)	7.2 (0.70 to 20.6)**	2.1 (0.26 to 25.1)
Api m 1/Ves v 5						
Before VIT	5.7 (<2 to 10.8)	1.6 (<1 to 12.6)	<4.6 (<4.6 to 14.0)	0.13 (<0.04 to 0.45)	0.24 (<0.07 to 2.3)	n.d.
1 month of VIT	6.9 (2.1 to 12.6)	1.9 (<1 to 13.9)	<4.6 (<4.6 to 15.2)	0.13 (<0.04 to 0.46)	0.41 (<0.07 to 2.9)	n.d.
2 months of VIT	12.7 (2.0 to 16.7)*	3.7 (<1 to 13.1)	<4.6 (<4.6 to 14.1)	0.11 (<0.04 to 0.46)	2.3 (<0.07 to 9.0)*	n.d.
1 year of VIT ^a	12.5 (6.8 to 40.9)*	3.2 (1.1 to 12.0)	<4.6 (<4.6 to 12.6)	0.14 (0.05 to 0.34)	4.5 (0.39 to 21.6)*	n.d.
CCD						
Before VIT	<2 (<2 to 4.2)	<1 (<1 to <1)	<4.6 (<4.6 to 4.6)	0.15 (<0.04 to 0.58)	<0.07 (<0.07 to 0.66)	n.d.
1 month of VIT	<2 (<2 to 4.2)	<1 (<1 to <1)	<4.6 (<4.6 to <4.6)	0.17 (<0.04 to 0.54)	<0.07 (<0.07 to 1.14)	n.d.
2 months of VIT	<2 (<2 to 4.4)	<1 (<1 to <1)	<4.6 (<4.6 to 4.7)	0.13 (<0.04 to 0.56)	<0.07 (<0.07 to 0.53)	n.d.
1 year of VIT ^a	<2 (<2 to 2.3)	<1 (<1 to <1)	<4.6 (<4.6 to <4.6)	0.13 (<0.04 to 0.20)	<0.07 (<0.07 to 0.50)	n.d.

^a $n = 6$. * $p < 0.05$; ** $p < 0.01$. Significant differences are compared to before VIT (Wilcoxon matched-pairs signed-rank test). n.d., not determined.

cept for specific IgG3, which showed a weak correlation that mainly was dependent on 3 high-concentration samples from 1 patient ($r_s = 0.6296$, $p < 0.01$).

Discussion

Knowledge about the natural history of IgG responses to foreign antigens has been hampered by the lack of reliable standardized subclass-specific antibody assays. Due to the wide range of methods used it is currently difficult to compare the levels of allergen-specific IgG across studies. ImmunoCAP represents a standardized quantitative immunoassay platform that is recognized as the gold standard in allergy diagnostics [18]. While the ImmunoCAP platform comprises assays for human IgE, IgA, IgG, and IgG4 antibodies, no ImmunoCAP assays for IgG1, IgG2, and IgG3 antibodies have so far been described. The goal of the present study was to develop assays for these 3 subclasses. Our preliminary characterization study showed that robust sensitive assays with high assay precision were developed for the measurement of antibodies of the IgG1 and IgG3 subclasses in serum or plasma. However, the new specific IgG2 assay showed a bias for the allotype G2m23, which required adjustment of the obtained assay results using compensation factors. One major drawback is that knowledge of the IgG2 allotype expression is then required, which is not always available. In the present study all samples were analyzed for G2m23 expression using a double-diffusion assay previously described [17]. Furthermore, the sensitivity of the specific IgG2 assay was lower than expected, probably due to a

low affinity of the IgG2-specific antibody used in the antibody-enzyme conjugate. The problem of finding high-affinity monoclonal antibodies specific for the human IgG subclasses, especially IgG1 and IgG2, is well known [11, 19]. Indeed, it was a challenge finding subclass-specific antibodies suitable for the ImmunoCAP technology that combined a high affinity with an appropriate specificity. Another problem was finding both high positive samples and truly negative samples for the validation studies. Thus, the matrix recovery studies, especially for the specific IgG1 assay, were problematic when background corrections had to be applied.

Most published studies regarding IgG subclass antibody responses do not show characterization/validation data for the assays used, making the interpretation of the results problematic. Moreover, in most cases no calibrator systems are applied, making it difficult to compare the individual IgG subclass responses due to presumed differences in the affinity of the detection reagents and other assay variations. The new research assays described in the present study utilize the standardized ImmunoCAP technology, initially developed for in vitro diagnostic use, including standardized assay reagents and the automated Phadia 100 instrument, which offers controlled incubation times and climate for the assay runs. The new assays for IgG1, IgG2, and IgG3 subclass antibodies were used together with the ImmunoCAP assays for specific total IgG, IgG4, and IgE to measure antibodies in serum/plasma samples.

The antibody response to BLG from milk in healthy, nonmilk-allergic adults was characterized by relatively low levels of pan IgG antibodies, which mainly belonged to the IgG4 subclass, and with a large interindividual vari-

ation in the antibody response. The IgG3 antibody levels were about 10 times lower than IgG4. Almost all subjects were negative for specific IgG1 and IgG2 antibodies. However, these subjects most probably still have significant IgG1 and/or IgG2 antibody levels to BLG, but below the cut-off values of the assays that were higher than for the IgG3 and IgG4 assays. This is supported by the fact that the serum levels of specific IgG3 and IgG4 corresponded to just 31% of the pan IgG antibody levels, indicating that IgG1 and/or IgG2 must contribute to the missing part of the IgG response to BLG. This conclusion could only be drawn because all 5 antibody assays report the results in mass units, i.e. their calibrators were all traceable to the WHO IRP 67/86 for human IgG, IgA, and IgM, for which the IgG subclass composition has been described [14, 15].

It has been speculated that the concentration of IgG3 in IRP 67/86 has been wrongly underestimated and other reference preparations for IgG are available with various reported subclass compositions [4]. However, since all commercially available ImmunoCAP antibody assays have calibrators traceable to recognized WHO IRPs, we decided to also use IRP 67/86 for the new IgG1, IgG2, and IgG3 antibody assays in order to compare the antibody concentrations.

The relatively high serum levels of IgG4 to BLG shown in the present study have also been indicated in previous studies [20, 21]. It is probably a consequence of repeated exposure to the antigen due to milk consumption leading to a strong IgG4 subclass antibody response in these tolerant individuals [7, 8]. Our study indicates that the IgG subclass response to BLG is stable over time since no major differences were observed in samples collected 85 days apart.

Currently, the clinical significance of food-specific IgG is not well understood. Studies in the area of food allergy have reported different findings and, whilst food-specific IgG4 has been shown to increase during successful oral immunotherapy [22, 23], there are conflicting data on whether or not specific IgG4 is higher in children with food allergy compared to nonallergic children. Recent data suggest that antibody responses may vary with the allergen, dose, and timing of exposure [20, 24–26]. Most studies on specific serum IgG responses to food proteins have investigated responses to egg, milk, and wheat proteins, which are 3 of the “big eight” food allergens, i.e. major causes of food allergy globally. More data on immune responses, including IgG antibody levels and subclasses, to a wider range of ingested proteins (including non- or weakly allergenic proteins) and over time is

required to gain insights into the development of tolerance or allergic responses (including factors involved in losing tolerance) at different times during life.

The antibody response to HDM, an important source of respiratory allergens, was characterized by very high levels of IgG antibodies in the group of subjects without known HDM allergy. The HDM-specific IgG4 response was very low, even lower than the IgG3 response. This is in accordance with the study by Kronqvist et al. [27]. Half of the subjects were IgE sensitized to HDM ($>0.1 \text{ kU}_A/\text{L}$), although their IgE antibody levels were low. This high sensitization rate might be explained by the selection of subjects with high IgG antibody levels and the sensitive ImmunoCAP assay.

Surprisingly, the measured HDM-specific IgG2 levels were higher than the IgG1 antibody levels. This IgG2 dominance in the IgG antibody response to HDM might be questioned considering the allotype bias of the IgG2 detection reagent of the new ImmunoCAP assay. However, the compensation factors used to adjust the measured specific IgG2 according to the allotype expression did not increase the reported IgG2 concentration for any subjects, but were used to decrease the reported concentration of subjects expressing the G2m(23) allotype since the calibrator IgG2 in the assay was of the nonallotype G2m(.) variant [13]. Furthermore, the overall sum of all IgG subclass antibodies was comparable with the measured levels of the HDM-specific pan IgG, although the individual variation was large. Even if the present study could be performed using allotype-dependent compensation factors, the detection antibody of the specific IgG2 assay should be replaced in the future when a more suitable IgG2-specific antibody can be obtained.

IgG2 antibody responses have previously been associated with bacterial polysaccharide antigens [5, 6]. However, strong IgG2 antibody responses to mite extracts have also been reported by others, as in our study [28, 29]. The role of IgG2 is believed to be similar to that of IgG4 in preventing inflammation and as being an isotype characteristic for later stages of an immune response [30].

All 8 bee/wasp-allergic subjects had measurable venom-specific IgG before starting VIT, and most of them also had measurable IgG1 antibodies but at lower levels. Only 3 of them had measurable venom-specific IgG2 and, since the assay cut-off was as high as $4.6 \text{ mg}_A/\text{L}$, the IgG2 antibody levels in these individuals were relatively high. The IgG3 and IgG4 antibody levels were low at the start of VIT, but later the venom-specific IgG4 increased dramatically, which is typical of successful VIT treatments [10, 31]. No major changes were generally ob-

served for the other IgG subclasses, but an increase in specific IgG1 was observed in some patients, as reported by others [32]. Only minor changes in specific IgE were seen in some of the patients. By using CCD-free recombinant allergens from bee (Api m 1) and wasp (Ves v 5), it was indicated that the majority of the IgG response was directed to these allergens previously recognized as major allergens [33]. Very little IgG antibody response was observed to CCD in these samples, mostly related to IgG3 and IgG4, and the response did not increase during VIT.

In conclusion, we have developed and performed a preliminary characterization of antibody assays for IgG1, IgG2, and IgG3 for research use based on ImmunoCAP technology. The assay runs are performed using the automated Phadia 100 instrument. The specific IgG1 assay has an LLOQ of 1.0 mg_A/L and the specific IgG3 assay has an LLOQ of 0.04 mg_A/L. The specific IgG2 assay with an LLOQ of 4.6 mg_A/L showed a bias for the IgG2 allotype G2m(23) and the measured results must therefore be recalculated using compensation factors according to the allotype expression. Preliminary studies of IgG subclass antibody concentrations using the new assays indicated a strong and stable IgG4 antibody response to BLG in healthy nonmilk-allergic individuals, a surprisingly high IgG2 antibody response to HDM in

subjects with high IgG antibody levels, and a more mixed IgG subclass response to venom allergens in bee/wasp-venom-allergic patients, which changed to a more pronounced IgG4 antibody response during VIT treatment. These new subclass-specific antibody assays for research use may be valuable tools for immunological studies, enabling comparative data on allergen/antigen-specific antibody profiles to be generated using a standardized approach and facilitating data interpretation across studies.

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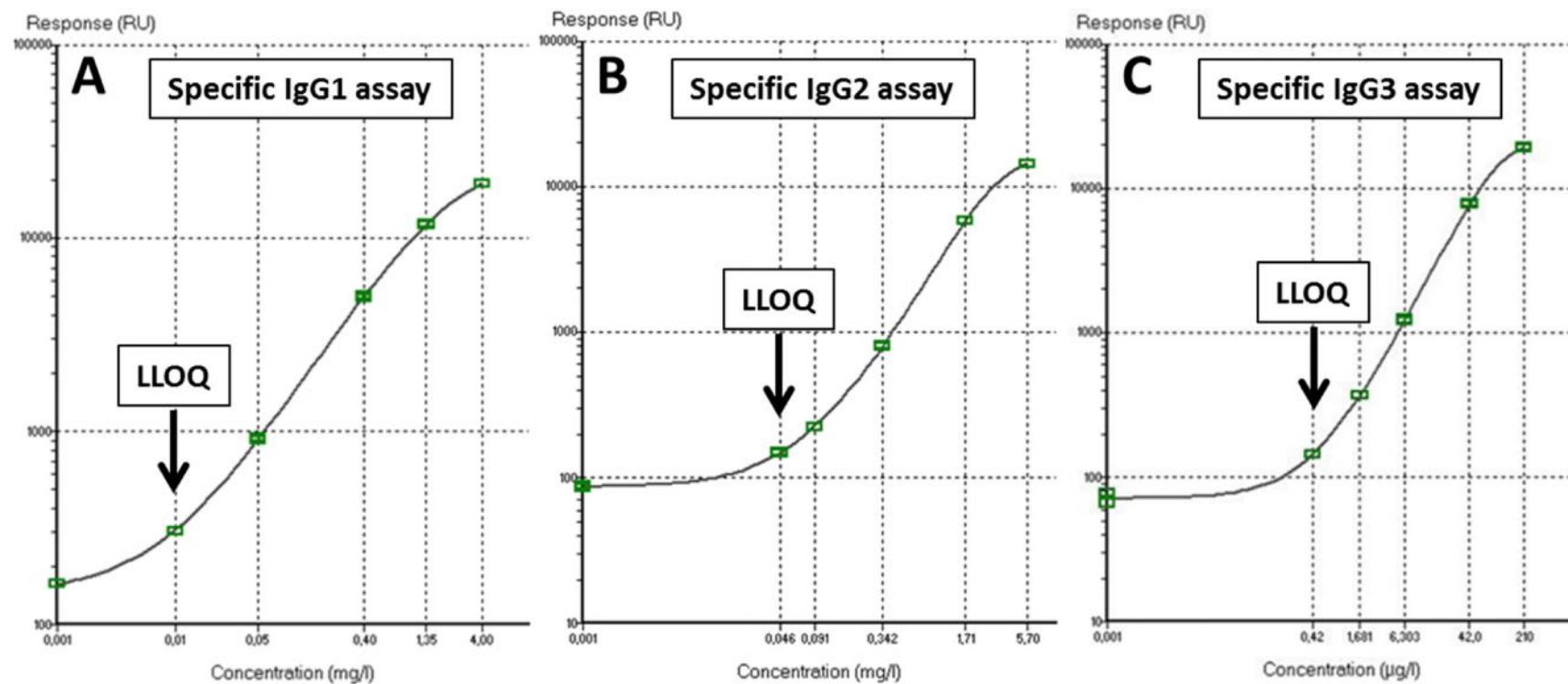
Disclosure Statement

Robert Movérare, Karin Blume, Peter Lind, and Åsa Marknell DeWitt are employees of Thermo Fisher Scientific. René Crevel and Stella Cochrane are employees of Unilever.

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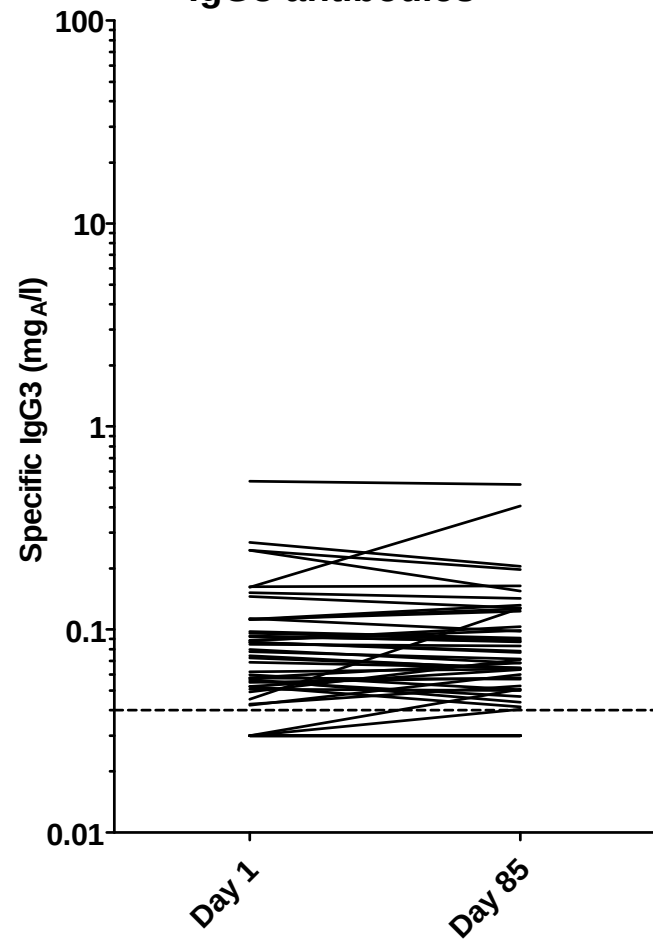
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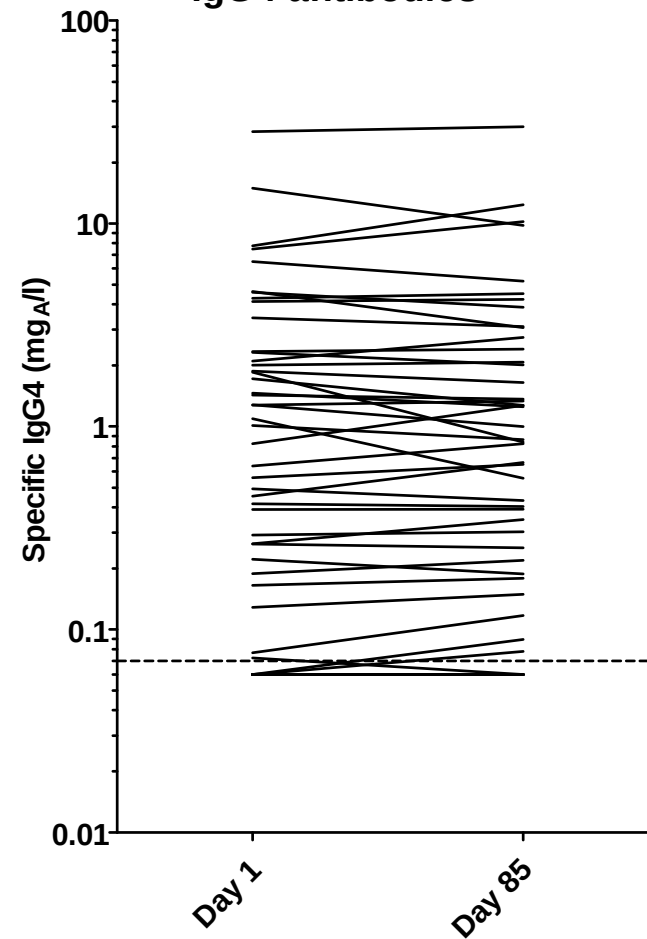
Online Suppl. Figure S1

Online Suppl. Figure S1. Calibration curves of the ImmunoCAP research assays for quantitative measurement of (A) IgG1 antibodies, (B) IgG2 antibodies, and (C) IgG3 antibodies in human serum or plasma samples. Lower limit of quantitation (LLOQ) is indicated.

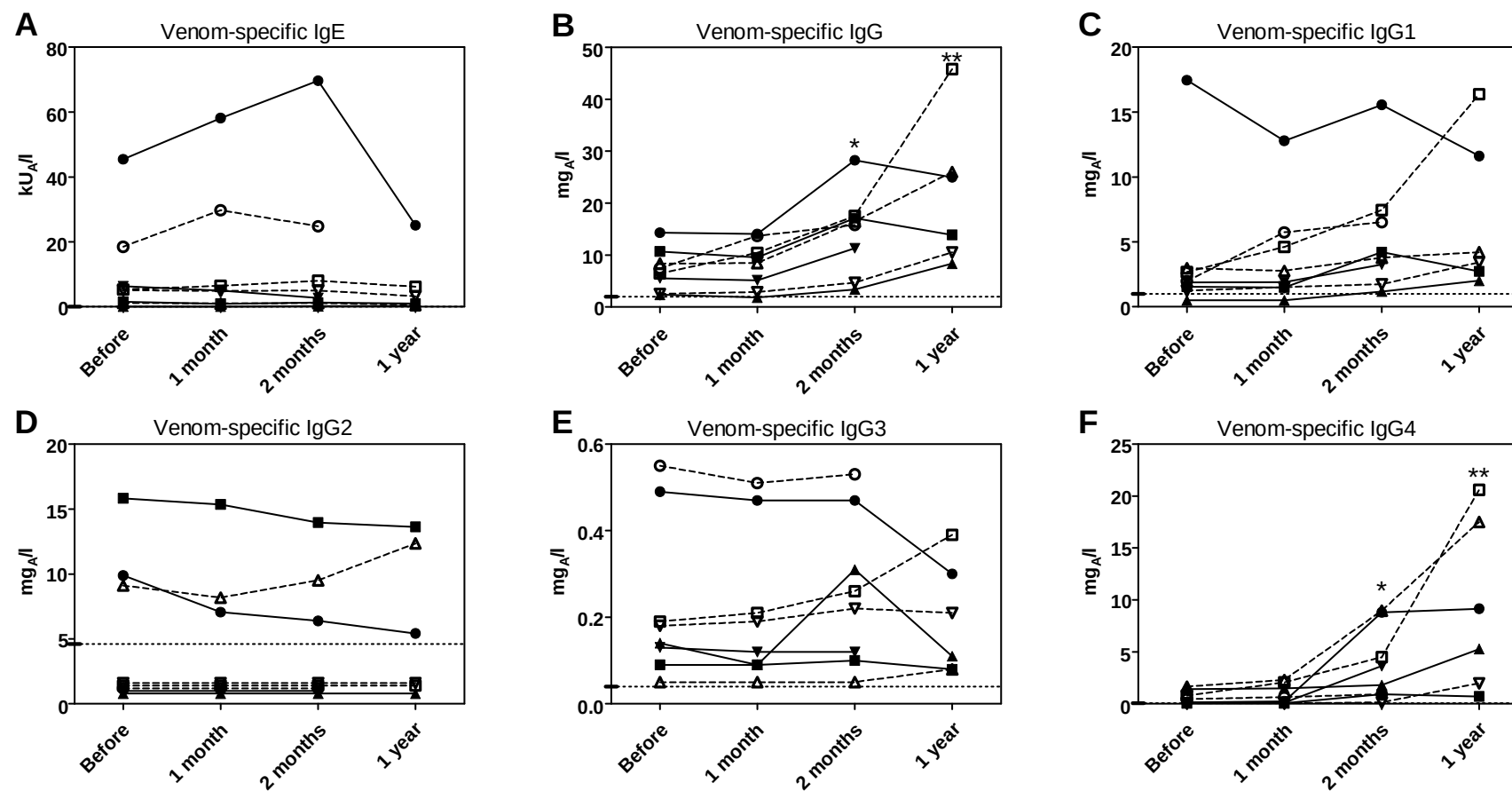
A Beta-lactoglobulin-specific
IgG3 antibodies



B Beta-lactoglobulin-specific
IgG4 antibodies



Online Suppl. Figure S2. Difference in serum concentrations of IgG3 antibodies (A) and IgG4 antibodies (B) to BLG in 2 samples collected 85 days apart from healthy subjects ($n = 56$). No significant differences in IgG3 and IgG4 antibody concentrations between days 1 and 85 were observed.



Online Suppl. Figure S3

Online Suppl. Figure S3. Venom-specific IgE (A), Pan IgG (B), IgG1 (C), IgG2 (D), IgG3 (E) and IgG4 (F) antibody concentrations during bee (n=4, open symbols) or wasp (n=4, closed symbols) venom immunotherapy. Lower limit of quantitation is shown as dotted line. Significant differences from baseline (Before) are indicated: * $P<0.05$, ** $P<0.01$