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Development of anti-PEG IgG/IgM/IgE ELISA assays for profiling anti-PEG immunoglobulin response in PEG-sensitized individuals and patients with alpha-gal allergy

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Abstract

Polyethylene glycol (PEG) is frequently used in various protein and nanomedicine therapeutics. However, various studies have shown that select PEGylated therapeutics can induce production of anti-PEG antibodies (APA), potentially culminating in rapid clearance from the systemic circulation, loss of efficacy and possibly increased risks of allergic reactions. Although IgE is a frequent cause of immediate hypersensitivity reactions (IHR), the role of IgE APA in PEG-related IHR is not well understood, due in part to a lack of standardized assays for measuring IgE APA. Here, we developed a rigorous competitive ELISA method to measure the concentrations of various APA isotypes, including IgE, with picomolar sensitivities. In a small number of serum samples from patients with known PEG allergy, the assay allowed us to detect a strong correlation

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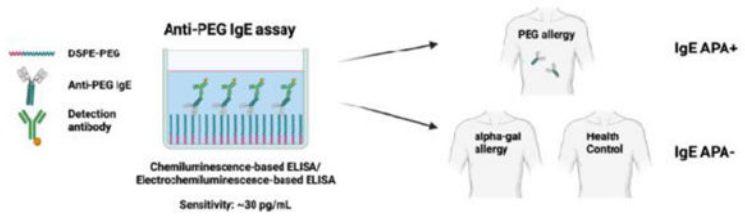
Declaration of Competing Interests:

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: S.K.L. and Z.L. are inventors of submitted patent application focused on methods to block APA and APA-related allergy. S.K.L. is also the founder and interim CEO for Polyon Pharmaceutical, a company that has licensed the technology, and has equity interests in Polyon. The terms of these arrangements are managed by UNC-CH in accordance with its conflict-of-interest policies.

between IgG and IgE APA in individuals with history of allergic reactions to PEG or PEGylated drugs, but not between IgM and IgE APA. We detected appreciable levels of IgG and IgM APA in individuals with history of alpha-gal allergy, however, they were not elevated relative to those detected in other healthy controls, and we found no pre-existing IgE APA. While preliminary and should be further investigated, these results suggest that differences in the route and mechanism of PEG exposure may drive variability in APA response.

Graphical abstract



Keywords

Polyethylene glycol (PEG); Anti-PEG immune reaction; Anti-PEG antibody; Hypersensitivity

Introduction

Polyethylene glycol (PEG), due to its hydrophilicity and flexibility, has been frequently employed to prolong the circulation and reduce the immunogenicity of many protein and nanoparticle therapeutics [1, 2]. Paradoxically, although PEG can shield and reduce the adsorption of opsonin to the underlying therapeutics, immune responses can be directed specially against PEG in the form of anti-PEG antibodies (APA) [3–6]. High titers of IgG and IgM class anti-PEG antibodies (IgG APA, IgM APA) can induce formation of APA/PEG-drug immunocomplexes that are quickly eliminated from the systemic circulation, leading to loss of efficacy. For instance, loss of efficacy was detected in around 40% – 90% of patients receiving Krystexxa® (PEG-uricase) and Oncaspar (PEG-asparaginase) [7, 8]. Worse, increased levels of APA are associated with increased risks for hypersensitivity, ranging from infusion reactions to life-threatening anaphylaxis [7–9]. Infusion reactions appear to be more prevalent in patients with drug-induced APA [8, 10]. Since IgE is a common mechanism of allergy, IgE class anti-PEG antibodies (IgE APA) are cited as a possible biomarker in cases of hypersensitivity reactions (HSR) to PEGylated drugs [11–14], including in cases of anaphylaxis to COVID-19 mRNA vaccines [15, 16]. Nevertheless, the precise role of IgE APA remains controversial, as it is also frequently not characterized or not detected in cases of allergic reactions to PEG, and patients with suspected IgE mediated reactions to PEG have been documented to tolerate COVID-19 mRNA vaccines [17].

One major contributing factor to the discrepancies in reported APA prevalence or concentrations is the lack of standardized methods for quantifying APA, and the lack of reporting in units that can be standardized across labs. Various detection methods of IgG and IgM APA have been developed, including passive hemagglutination [18], double antigen bridging assay [19], dual cytometric beads assay [13], enzyme-linked immunosorbent assay

(ELISA) [5, 20, 21], and flow cytometry [22]. However, the variations in detection methods can create major inconsistencies in reporting the prevalence and the levels of different APA isotypes. Not surprisingly, the reported prevalence of APA in the general population varies from 0.2% to 72% [20, 23]. Even data generated using the same approach (e.g., ELISA) may not be comparable across different labs, as most ELISA studies do not include APA standards to report the exact APA concentrations. Instead, those studies simply report relative differences (e.g., change in endpoint dilution), where the results can vary substantially due to differences in experimental procedures, equipment, and lack of quality controls of reagents. Endpoint dilution-based measurements are also susceptible to small variances at the lower limit of detections (LLOD). These experimental variations make it difficult to elucidate the actual levels of APA in humans necessary to trigger loss of efficacy and increased risks of hypersensitivity.

To overcome these challenges, we described here an optimized competition ELISA format that can report in well-standardized mass concentrations, the specific levels of different APA isotypes, including sensitive detection of IgE APA down to tens of pg/mL. Using these assays, we discovered that individuals with history of alpha-gal allergy possess pre-existing IgG and IgM APA, but not IgE APA, which appears to be primarily drug-induced.

Methods

Absorbance-based IgE APA ELISA

50 μg/mL DSPE-PEG 5000 (NANOCS, PG1-DS-5k) was incubated in non-treated half-area 96-well plates (Corning, 3695) overnight at 4°C. The plates were washed with PBS three times and blocked with 5% non-fat milk (Lab scientific bioKEMIX, M0841) for 1 hour at room temperature. Two human anti-PEG IgE standards, 6.3 and 15–2b, which specifically bind to the PEG backbone or methoxy end group of methoxy PEG, respectively, were prepared. The 6.3 IgE antibody (anti-PEG, Hu-6.3-IgE) was obtained from Academia Sinica, while the 15–2b IgE was synthesized in our lab, which involved grafting the Fab domain containing the variable sequence of human 15–2b IgG to the constant region of a human IgE antibody. Standard antibodies were serial diluted in 1% milk and added in triplicate to blocked 96-well plates. In parallel, samples were diluted 10-fold, in 1% milk or 1% milk + 10 mg/kg 10 kDa mPEG, and added in triplicate, followed by 2-hour incubation on the shaker at room temperature and six times washing. Subsequently, mouse anti-human IgE Fc-HRP (SouthernBiotech, B3102E8) was added and incubated for 1 hour, followed by another six-time washing. TMB substrate (ThermoFisher, 34029) was then added to the plates. After ~5 min incubation, protected from light, 1N HCL was added to stop the reaction. The absorbance at 450nm and 595nm of each well was measured by accuSkan FC microplate photometer (Fisher, 14-377-576). A detailed step-by-step protocol is provided in the Supplementary Materials.

Chemiluminescence-based IgE APA ELISA

The chemiluminescence-based ELISA was similar to absorbance-based ELISA except the following parameters. DSPE-PEG was coated on full-area white-bottom 96-well plates (Corning, 3922). Instead of TMB, Femto substrates (ThermoFisher, 37075) were added to

the plates, and the luminescence signal of each well was directly scanned by SpectraMax iD3 (SpectraMax iD3, Molecular Devices). A detailed step-by-step protocol is provided in the Supplementary Materials.

Electrochemiluminescence-based IgE APA ELISA

The electrochemiluminescence-based ELISA was similar to absorbance-based ELISA except the following parameters. DSPE-PEG was coated on the MULTI-ARRAY® 96-well plates (L15XA-3, MSD). After incubation with anti-PEG IgE standard and samples, detection antibody (F215A-3, MSD) was added to the plate. After one-hour incubation at room temperature and six times washing with PBS, MSD read buffer T, surfactant free (R92TD-2, MSD) was added to each well. The luminescence signal was then measured with MESO QuickPlex SQ 120MM (MESO QuickPlex SQ 120MM, MSD). A detailed step-by-step protocol is provided in the Supplementary Materials.

Determination of sensitivity

The sensitivity of each measurement was determined by signals in the blank wells and standard curves. The minimum detectable signal equals average of signal in blank wells plus three times the standard deviation of signals of blank wells [24]. The minimum quantitative signal equals average of signal in blank wells plus 10 times the standard deviation of signals of blank wells. The LLOD and lower limit of quantitation (LLOQ) were the correlated concentration of minimum detectable signal and minimum quantitative signal based on the standard curves, respectively.

Quantitation of IgG, IgM and IgE APA in samples

Blood samples with IgE APA (n=6) and negative controls (n=9) were acquired from Vanderbilt University Medical Center (Drs. Elizabeth J. Phillips and Cosby A. Stone). Blood from patients with history of alpha-gal allergy was acquired from UNC (Dr. Scott P. Commins) (n=41). With aforementioned methods, IgG, IgM and IgE levels of each sample were assessed with standard antibodies. Human 6.3 IgG (anti-PEG, Hu-6.3-IgG) and human AGP4 IgM (anti-PEG, cAGP4-IgM) were obtained from Academia Sinica. We characterized the molecular weight by SDS-PAGE together with the IgE standard (Supp Figure 1). The binding affinity was measured using ForteBio Octet Red384 equipped with streptavidin sensors (Sartorius, 18–5019) and biotin-PEG (CreativePEGWorks, PLS-2054) (Supp Table 1). For IgE measurement, both CL- and ECL-based ELISA were performed. Two independent experiments were performed with the average reported. The whole study was double-blinded. Differences between groups were determined by nonparametric Mann-Whitney tests and correlation was determined by nonparametric Spearman's correlation using the GraphPad Prism software (GraphPad Software Inc., USA). Significance was considered as statistical analysis value less than 0.05 ($p < 0.05$) between groups. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$.

Results

Optimization of IgE APA ELISA

The level of IgE in blood is much lower than that of IgG and IgM. This necessitates developing an assay with much higher sensitivity than what we had previously developed for IgG and IgM APA [20]. We first assessed the sensitivity of conventional ELISA that relied on detecting differences in absorbance from conversion of colorimetric substrates by horseradish peroxides (HRP) on IgE APA measurement. The absorbance-based ELISA yielded a LLOD at ~0.45 ng/mL and a LLOQ at ~1 ng/mL for IgE APA (Figure 1A). We included two different APA standards in the study, with 15–2B specifically binding to the methoxy end group of methoxy PEG, and 6.3 binding to the PEG backbone. We observed no appreciable difference in APA levels estimated based on 6.3 *vs.* 15–2B standards. The level of IgE APA detection sensitivity is comparable to assays quantifying IgG and IgM APA standards (data not shown). Given there is ~ 300 ng/mL total IgE in human body [25, 26], we concluded the sensitivity from absorbance-based ELISA was unlikely to be adequate for IgE APA.

We next assessed two other ELISA methods, based on measuring either the chemiluminescence (CL) from HRP-conversion of light-emitting substrate, or the electrochemiluminescence (ECL) induced from a specific electron-involved reaction. The electrochemiluminescence method involves using microplates with carbon electrodes in the bottom, and detection by secondary antibodies with SULFO-TAG, replacing HRP-bound secondary antibodies used in absorbance-based ELISA. During reading, electricity was applied to the plate electrodes by an MSD instrument, leading to a light emission reaction. Each method was optimized (Supp Figure 2 and 3), and confirmed the absence of cross-reactivity with other reagents used in the ELISA (Supp Figure 4). The electrochemiluminescence-based ELISA yielded a LLOD of ~ 14 pg/mL and a LLOQ of ~ 30 pg/mL, and the chemiluminescence-based ELISA yielded a LLOD of ~ 4 pg/mL and a LLOQ of ~ 37 pg/mL (Figure 1B and 1C). Both of the ELISA formats offered ~ 30-fold greater detection sensitivity than conventional absorbance-based ELISA. We observed similar sensitivity and signal intensity regardless of whether the IgE APA standard bound to methoxy-PEG end group or the PEG backbone. We utilized 6.3 (binding to PEG-backbone) as the APA standard in all remaining assays.

Assessing APA in individuals with history of drug-induced PEG allergy:

We tested if our assay could measure the APA levels in serum samples with previously confirmed IgE APA, as well as negative controls [11, 27]. We were able to detect IgE APA in 100% of serum samples previously found to have detectable IgE APA, with an average level of ~21.4 ng/mL (Figure 2A). Both CL and ECL-based ELISA yielded similar results (Supp Figure 5). As a validation of our detection specificity, we detected no IgE in any of the negative serum controls.

We then investigated the relationship among different APA isotypes. While the level of IgM APA was comparable between those with detectable IgE APA (0.67 ug/mL) and controls (2.14 ug/mL), the levels of IgG APA in patients positive for IgE APA (8.44 ug/mL) were

substantially higher than those from IgE APA negative controls (0.75 ug/mL). In all of the examined samples, IgE APA were consistently found together with *both* IgG and IgM APA (Figure 2B). However, in the absence of IgE APA, IgG and IgM APA can occur independently. We further analyzed for any potential correlation between anti-PEG IgG, IgM and IgE concentrations. Interestingly, we found a strong correlation between IgG and IgE levels ($r = 0.7293$, $p = 0.0030$), while no correlation was found between IgG and IgM levels, as well as between IgM and IgE levels (Figure 3).

To evaluate the relevance of our IgE APA measurements, we further investigated the association with the clinical presentation of the patients (full description available in Supp Table 2). We found high IgE APA levels (>10 ng/mL) in all patients with a history of severe anaphylaxis (WAO (World Allergy Organization Systemic Allergic Reaction Grading System) Grade 5), and either low or non-detectible levels of IgE APA in patients with milder immediate symptoms less suggestive of an IgE mediated reaction (WAO Grade 2) (Table 1). No IgE APA was found in healthy controls without a history of PEG allergy. All patients with IgE APA were positive to skin tests against PEG and/or PEG containing products.

Pre-existing APA in patients with alpha-gal allergy

Hypersensitivity against COVID-19 mRNA vaccines was more frequently reported in patients with a history of allergy, with a number of reports suggesting a link between APA and allergic reactions to COVID mRNA vaccines. We thus sought to assess the levels of various APA immunoglobulins in patients with history of IgE-mediated allergy to alpha-gal, as a control allergy. We acquired a panel of 31 samples from individuals with history of alpha-gal allergy, a food allergy mediated by IgE, as well as 12 healthy controls. We were able to detect the presence of IgG and IgM APA in 61% and 97% of the alpha-gal IgE+ specimens, respectively. We didn't detect any IgE APA in these samples (Figure 4A). The prevalence of alpha-gal allergic patients with detectable levels of APA is consistent with the prevalence of APA previously reported [20], and also the prevalence detected among the healthy controls in this study, where IgG and IgM APA were both found in ~83% of healthy control.

Despite the prevalence, the amount of APA remained quite low: the average IgG and IgM APA were 0.03 μg/mL and 0.13 μg/mL, respectively, in alpha-gal IgE+ patients. This compares with the average IgG and IgM APA were 0.17 μg/mL and 0.43 μg/mL, respectively, in healthy controls and the modest differences were not statistically significant. Only a small fraction of alpha-gal IgE+ samples possessed IgG APA > 1μg/mL (6%) or IgM APA > 1μg/mL (16%) (Figure 4B and 4C). However, more specimens from healthy controls possessed high APA levels (16% for IgG and 42% for IgM).

Discussion:

Due to its simplicity and minimal requirement of equipment, ELISA is one of the most frequently employed methods for measuring antibody levels. However, the conventional absorbance-based ELISA methods did not offer sufficient sensitivity for IgE APA measurement. Here, we reported chemiluminescence and electrochemiluminescence-based competition ELISAs, both of which can be employed for more sensitive IgE APA

measurements. The two methods offer similarly high sensitivity, and are able to reliably detect IgE APA with ~30-fold greater sensitivity than traditional absorbance-based ELISA. The improved sensitivity was essential in detecting drug-induced IgE APA, and confirming the lack of IgE APA in individuals with history of alpha-gal allergy and corresponding healthy controls. We are also able to quantify, for the first time, the levels of drug-induced IgE APA (avg ~21ng/mL).

To date, most studies assessing PEG-immunogenicity do not employ assays with APA standards that enable accurate quantification of actual APA concentrations. Instead, they are frequently assayed by comparing relative differences to controls, such as in detection by endpoint dilutions. While sufficient to support a particular scientific conclusion within a study, the actual dilution values from endpoint dilution assays vary substantially from lab to lab, due to differences in experimental protocols, equipment and reagents. Furthermore, by focusing on readout near the limit of detection rather than signals far above the detection threshold, endpoint dilution results are also more variable. Consequently, it is difficult to compare across different studies, particularly if there are inherent differences in the APA levels in the control population. Other assays (e.g., flow cytometry) generally are even less readily available, and/or are difficult to standardize among different research groups due to differences in instrumentation [28]. Such assays are inherently difficult to compare results across different labs. These limitations also make it difficult for researchers and physicians alike to predict whether a specific patient possesses sufficient APAs that would greatly increase risks of adverse reactions or compromised efficacy. In contrast, by combining well-characterized APA isotype standards with a quantitative competition ELISA format, we can readily quantify various APA isotypes in each biospecimen in specific mass (ng/mL) or molar concentrations [29–31]. By using the same APA isotype standard antibodies and assay procedures, the resulting APA measurement can be readily compared and standardized by multiple research groups around the world. While measurements of clinical biospecimens intended to support regulatory filing and registration requires assay qualification and measurements in CLIA-certified lab setting that are ill suited for most academic research labs, the bar for standardizing assays for academic investigation of clinical specimens is far lower, and the current protocols described in this work is sufficient. Indeed, the protocol described here is used to measure APA levels for specimens from a Phase 2 clinical trial (NCT04761822).

While the IgM APA concentrations detected in healthy controls in this study were quite low as expected, they were somewhat higher than what we had previously reported for the general population [20]. We believe this may be partially attributed to a number of factors. First, given the substantial variations of APA among the general population, the differences could simply reflect the limited sample size here. Second, as our reported values are normalized to an APA standard, differences in the affinity of the APA standard towards PEG, either from inherent batch-to-batch variations or different sources altogether, may proportionally skew the assay outputs. Indeed, normalizing the absorbance or luminescence signal to an Ab standard with weaker affinity would lead to a higher estimated concentration. The anti-PEG IgM standard utilized in this study had a modest, roughly 3-fold weaker affinity than the IgM standard utilized in our earlier study, which in turn would account for the bulk of the difference in the higher APA readout (Supp Table

1). This underscores that, while it is important to include an APA standard to facilitate standardization of the assay output, it is equally important to characterize and report the binding affinity of the APA standard.

Currently, both IgE-mediated and non-IgE mediated reactions against PEGylated drugs have been reported [13, 32–34]. In IgE-mediated cases, mast-cell activation and basophil activation were observed; in non-IgE mediated cases, hypersensitivity commonly involves activation of complement system, commonly referred to as complement activation-related pseudoallergy (CARPA) [34, 35]. Here, we found IgE APA+ patients with severe anaphylaxis all possessed high IgE concentrations, while patients with milder symptoms compatible with anaphylaxis typically present only low levels of anti-PEG IgE. This suggests a potential relationship between severity of anaphylaxis and IgE concentrations. Skin-prick test (SPT) and intradermal testing (IDT) can be a practical and easy method to detect IgE APA in anaphylaxis patients, given that anaphylaxis patients with IgE APA had consistently positive prick tests SPT and/or IDT to PEG reagents. However, one anaphylactic patient without detectible IgE APA was negative with the SPT. Given the limited number of samples we analyzed, we believe it is too early to draw any definitive conclusions, however, this could define an alternative mechanism.

Interestingly, while we were able to detect appreciable levels of pre-existing IgG and IgM APA in individuals with alpha-gal allergy, we found no detectable levels of IgE APA at all. This contrasts sharply with our measurement of sera from patients who developed allergic responses against PEGylated drugs, where IgE APA was consistently detected. These results suggest that pre-existing IgE APA is relatively rare, and that IgE APA may most likely induced by specific exposure to PEG and/or PEGylated drugs, rather than other daily environmental exposures. Future studies will be needed to further confirm this hypothesis, and to better elucidate the specific mechanisms of induction of IgE APA and their role in allergic reactions to PEGylated drugs. The ELISA format reported here provided a strategy to clearly distinguish IgE vs. non-IgE mediated cases of HSR against PEGylated drugs, including the correlation between specific IgE APA and relative risks of HSR.

APA represents an essentially new class of anti-drug antibodies (ADA) with important emerging polypharmacy implications. Polypharmacy refers to the potential adverse effects of taking multiple medications concurrently, while further underscores the importance for the field to gravitate forward well-standardized assays of reporting. Traditionally, ADAs are by definition drug-specific, and occur only after repeated use of a particular drug. However, the chemical structure of PEG backbone (which most APA binds to) is identical between different PEGylated drugs. Thus, it is not surprising that APA stimulated or induced by one PEGylated drug (e.g., PEG-lipoplexes) can render a second PEGylated drug that otherwise shares few structural similarities (e.g., PEG-protein) non-efficacious or even unsafe. For instance, two patients treated with pegvaliase-pqpz (Palynziq®, and thus likely APA+ [32]) experienced serious adverse events (SAE) to progesterone that contained polysorbate with PEG motifs [36]. Likewise, we had recently described a case report of a patient with prior anti-PEG immunity experiencing an allergic reaction to the Moderna (SpikeVax®) COVID-19 vaccine [37]. The clinical impact of COVID-19 mRNA vaccines on the development of APA and potential impact on treatment with PEGylated medications

should be studied further. In one study, increase of both anti-PEG IgG and IgM were detected in patients receiving vaccines, and anti-PEG antibody level in patients with HSRs were even higher [38]. In another study, Moderna SpikeVax COVID-19 vaccine appeared to induce substantially greater APA response than the Pfizer Comirnaty COVID-19 vaccine [39]. The same study also found significantly higher association of Onpattro, a PEGylated nanoparticle drug for treatment of polyneuropathy, to blood phagocytes (granulocytes and monocytes) in patients after vaccination of mRNA COVID-19 vaccines, with greater binding at higher IgG and IgM APA. Similar results were also shown against doxorubicin liposome injection® (Doxil), a PEGylated liposome for cancer treatment [39]. Though the clinical significance of this is currently unknown, we anticipate polypharmacy issues associated with the induction of APA by different PEGylated drugs will inevitably become more prevalent as the number of approved PEGylated drugs increases and their use becomes correspondingly more widespread. Indeed, in addition to the ~30 FDA-approved drugs that contain PEG, a search of clinicaltrials.gov for interventions with the keyword “PEG” revealed at least 60 active (finished recruiting) trials involving a PEGylated therapeutic, and an additional ~200 open studies (not yet recruiting, recruiting, or available for expanded access). To most efficiently compare findings, it is imperative for the field to increasingly employ methods for sensitive APA measurement with unified standards.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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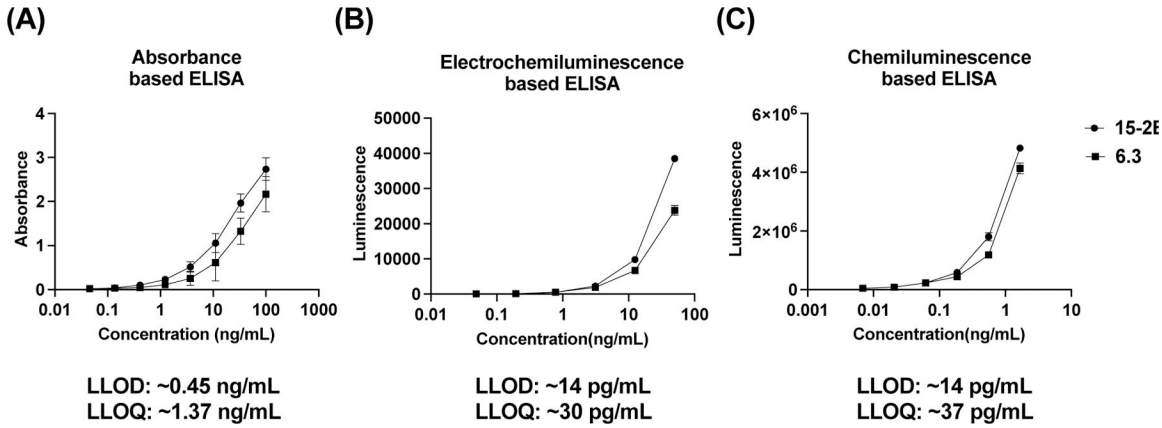
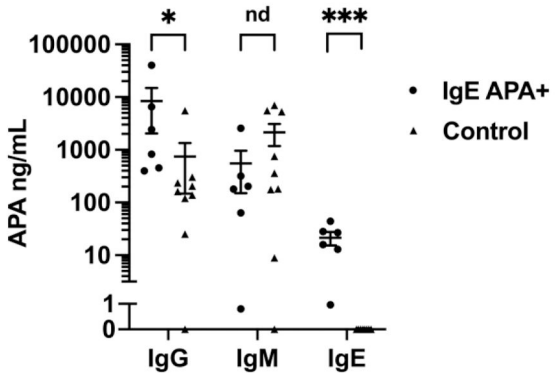


Figure 1. Quantitation of anti-PEG IgE using different ELISA formats.

Binding signal measured as function of different concentrations of anti-PEG IgE, detected using: **(A)** absorbance (Abs), based on conversion of TMB by HRP-linked secondary antibody; **(B)** electrochemiluminescence (ECL), based on the amount of sulfate-linked secondary antibody bound, and **(C)** chemiluminescence (CL), based on conversion of Femto substrate by HRP-linked secondary antibody. LLOD and LLOQ for each assay are listed. 15–2B IgE: binding with methoxy end group of mPEG; 6.3 IgE: binding with PEG backbone

(A)



(B)

	IgE+	Control
IgG only%	0.0	12.5
IgM only%	0.0	12.5
IgE only%	0.0	0.0
IgG and IgM only%	0.0	75.0
IgG and IgE only%	0.0	0.0
IgM and IgE only%	0.0	0.0
IgG, IgM and IgE%	100.0	0.0

Figure 2. Quantification of anti-PEG antibody isotypes in samples with known IgE and control.

(A) APA levels in patients with IgE APA and IgE-control. (B) Prevalence of different isotypes of APA among all samples: IgG only, IgM only, IgG and IgM only, IgG and IgE only, IgM and IgE only, or IgG, IgM and IgE.

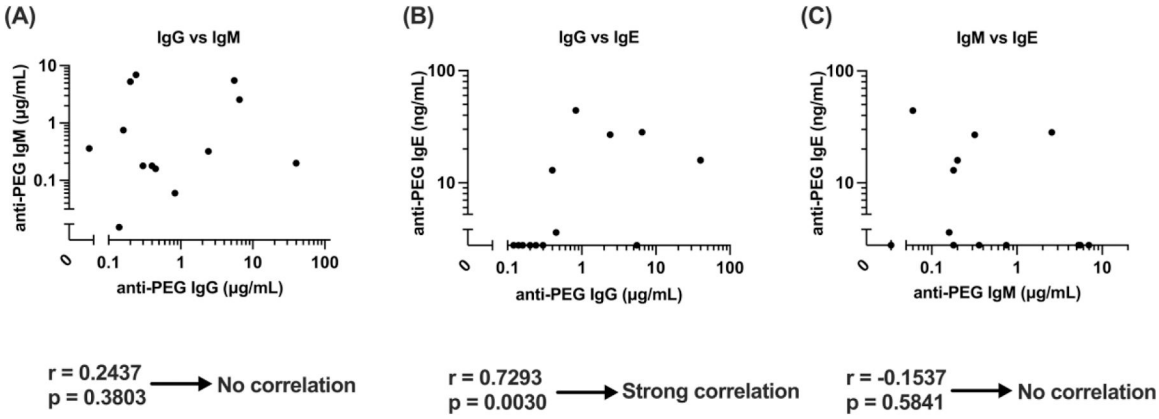


Figure 3. Correlation of IgG, IgM, and IgE APA in PEG-drug treated patients.

The correlation between (A) IgG and IgM APA, (B) IgG and IgE APA, and (C) IgM and IgE APA.

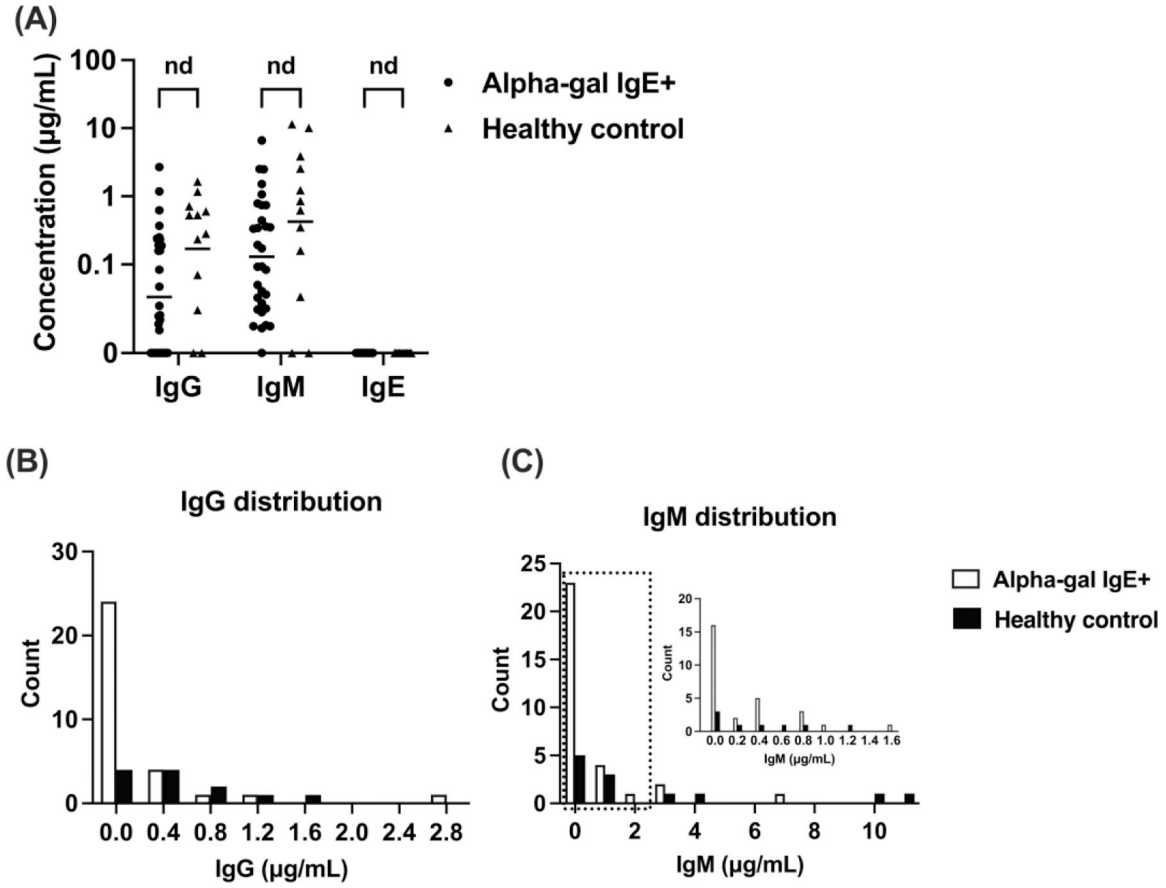


Figure 4. Pre-existing anti-PEG IgG and IgM levels in allergic individuals.

(A) Anti-PEG IgG and IgM levels in alpha-gal positive patients and healthy control. Anti-PEG IgE was not detectable. Distribution of (B) IgG and (C) IgM APA.

Table 1.

APA levels in patients with anaphylaxis induced by PEG-drugs

Sample ID	IgE (ng/mL)	IgG (μg/mL)	IgM (μg/mL)	Anaphylaxis	Skin test
1	15.89	>40	0.20	S	Y
2	0.68	0.45	0.16	M	Y
3	12.96	0.40	0.18	S	Y
4	26.82	2.42	0.32	S	Y
5	28.23	6.52	2.56	S	Y
6	44.23	0.83	0.06	S	Y
7	0.00	5.53	5.52	M	N

Anaphylaxis. S: severe anaphylaxis (Determined as at least two times of WAO Grade 5 anaphylaxis); M: mild anaphylaxis (Determined by WAO Grade 2 anaphylaxis)

Skin test for PEG sensitivity. Y: Positive to skin test against PEG and/or PEG containing product; N: negative to skin test against PEG and/or PEG containing product

All samples are from patients who developed anaphylaxis by PEG-drug. Sample 1–6 are from patients with IgE APA, and sample 7 is from a patient without IgE APA. Cells with high APA levels (> 10ng/mL IgE APA or >1 μg/mL IgG and IgM APA) were labeled with red.